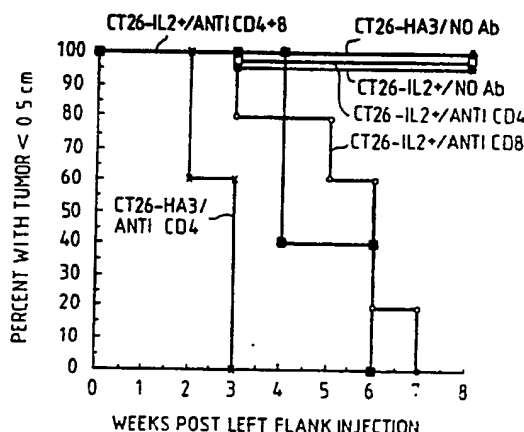




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 5 :</b> C12N 15/85, A61K 48/00, 35/12 C12N 5/10 // C12N 15/19 C12N 15/31, 15/54	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 92/05262</b>  <b>(43) International Publication Date:</b> 2 April 1992 (02.04.92)
<b>(21) International Application Number:</b> PCT/US91/06612 <b>(22) International Filing Date:</b> 12 September 1991 (12.09.91)  <b>(30) Priority data:</b> 582,711 14 September 1990 (14.09.90) US  <b>(71) Applicants:</b> THE JOHN HOPKINS UNIVERSITY [US/US]; 550 North Broadway, Baltimore, MD 21205 (US). BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West Seventh Street, Austin, TX 78701 (US).  <b>(72) Inventors:</b> FROST, Philip ; 8114 Meadow Crest, Houston, TX 77071 (US). VOGELSTEIN, Bert ; 3700 Breton Way, Baltimore, MD 21208 (US). CHERNAJOVSKY, Yuti ; 14A Rowsley Avenue, London NW4 1AJ (GB). FEARON, Eric, R. ; 3700 North Charles Street, #904, Baltimore, MD 21218 (US). PARDOLL, Drew ; 4448 Roland Springs Drive, Baltimore, MD 21210 (US).	<b>(74) Agents:</b> PATTERSON, Melinda, L. et al.; Arnold, White & Durkee, 750 Bering Drive, Suite 400, Houston, TX 77057 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

**(54) Title:** METHODS AND COMPOSITIONS FOR GENETIC THERAPY AND POTENTIATION OF ANTI-TUMOR IMMUNITY

**(57) Abstract**

The invention provides a preparation of tumor cell lines containing an exogenous gene encoding a polypeptide that potentiates the immune response to the tumor, for example, interleukin-2. The invention includes methods for cancer therapy by immunization with the immunopotentiating tumor cell. The invention also provides methodology whereby one can selectively ablate in *in vitro* and *in vivo* genetically altered cells. A preferred embodiment of this aspect of the invention includes a cell containing a first exogenous gene encoding an immunopotentiating polypeptide and a second exogenous gene encoding a "lethal" or "suicide" polypeptide, preferably under the control of an inducible promoter. Introduction of the second exogenous gene confers the ability to selectively kill the tumor cell by inducing a promoter in operative linkage to the gene encoding the lethal polypeptide to initiate transcription of the polypeptide. Diphtheria toxin or the thymidine kinase of Herpes simplex virus are exemplary lethal genes. An exemplary promoter employed is the 6-16 promoter, which is inducible with low levels of interferon. Methods for using the cells in cancer therapy are also provided.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU <sup>+</sup>	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE <sup>+</sup>	Germany	MC	Monaco	US	United States of America
DK	Denmark				

<sup>+</sup> Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

-1-

5

10

**METHODS AND COMPOSITIONS FOR GENETIC THERAPY  
AND POTENTIATION OF ANTI-TUMOR IMMUNITY**

15 This invention relates generally to the field of  
immunotherapy of cancer, and more specifically, to  
preparation of tumor cell lines containing an exogenous  
gene encoding a polypeptide that potentiates the immune  
response to the tumor, for example, interleukin-2. The  
invention includes methods for cancer therapy by  
20 immunization with the immunopotentiating tumor cell.

The invention also provides methodology whereby one  
can selectively ablate in vitro and in vivo genetically  
altered cells. A preferred embodiment of this aspect of  
the invention includes a cell containing a first exogenous  
25 gene encoding an immunopotentiating polypeptide and a  
second exogenous gene encoding a "lethal" or "suicide"  
polypeptide, preferably under the control of an inducible  
promoter. Introduction of the second exogenous gene  
confers the ability to selectively kill the tumor cell by  
30 inducing a promoter in operative linkage to the gene  
encoding the lethal polypeptide to initiate transcription  
of the polypeptide. Diphtheria toxin or the thymidine  
kinase of Herpes simplex virus are exemplary lethal genes.  
An exemplary promoter employed is the 6-16 promoter, which  
35 is inducible with low levels of interferon. Methods for  
using the cells in cancer therapy are also provided.

Active immunotherapy is considered to be a promising  
approach to the treatment and particularly to the  
prevention of recurrences of human cancer. Specific

active immunotherapy, one of the most promising approaches under investigation, involves activation of the host immune response against the tumor by immunization with tumor cells (which may be altered by mutagenesis, by treatment with a hapten, or by expression of foreign proteins) in order to activate specific effector cells of the immune system, such as cytolytic T lymphocytes. Nonspecific active immunotherapy may utilize microbial or chemical immunomodulators to activate natural killer (NK) cells, macrophages or lymphokine activated killing (LAK). Unfortunately, much of the promise of these approaches remains unfulfilled.

One of the most critical questions in cancer immunology is why the immune system fails to eliminate tumors. In the 1970's, Hewitt articulated the notion that most tumors did not express any tumor-specific or neoantigens and thus could not be recognized as "foreign" by the immune system. Indeed, virtually no tumor cell surface antigens recognized by antibodies were found to be tumor specific, and furthermore, most spontaneous murine tumors were considered "poorly immunogenic" as defined by their failure to be eliminated when transferred into syngeneic hosts (Hewitt et al., 1976). However, these same tumors could be rendered "immunogenic" by mutagenesis (Van Pel and Boon, 1982) when new antigens are expressed on the tumor cell surface.

It is possible that the immune system fails to eliminate tumors not because neoantigens are absent, but rather because the response to these neoantigens is inadequate. Therefore, a method for enhancing immunogenicity of the tumor cells so as to potentiate the host's immune response would provide a key advance in immunotherapy.

Failure to respond to tumor neoantigens may be due, at least in part, to a failure of T cell help. The molecular basis for Th function is the local secretion of

lymphokines, such as interleukin-2 (IL-2), that act upon CTLs whose T cell receptors have first been engaged by the appropriate antigen-MHC complex (reviewed in Moller, 1980). The cytotoxic potential of NK and LAK cells is also enhanced by IL-2 (Grimm et al., 1982; Phillips and Lanier, 1986; Ortaldo et al., 1986). Although potentiation of tumor immunity by systemic injection of interleukin 2 has been attempted, those studies were hampered by the toxicity of the systemically administered IL-2. Therefore, a method for potentiating immunity to tumors by providing accessory T cell help in the location of the tumor is a more attractive option, which has long been needed for cancer therapy.

An additional difficulty in immunotherapy results from the problems inherent in administration of a living neoplastic cell to a patient. In the past, tumor cells used for immunization were treated prior to immunization to reduce their proliferative potential, e.g., by irradiation or treatment with mitomycin C. Unfortunately, either one of these methods of inhibiting replication also significantly diminishes the immunogenicity of the cells. It has been shown that mutagen induced variants that were irradiated with 8-10,000 Rads are no longer immunogenic (Sella, 1989; Boon, 1985). Similarly, murine tumor cells secreting IL-2 or IFN- $\gamma$  lose their immune potential after irradiation. In addition, attempts at using membrane preparations of tumor cells also fail to produce convincing evidence of an immune response. It would therefore be advantageous to develop a means for using viable immunogenic tumor cells that could be deleted after they have induced an immune response.

The present invention provides for a novel immunopotentiating tumor cell variant, capable of inducing a host immune response to tumor antigens, comprising a selected immunopotentiating gene, for example, interleukin-2. The invention also provides a variant

comprising both an immunopotentiating gene and a lethal gene system.

In a first general embodiment, the invention provides a cellular preparation for potentiating the immune response to a tumor by a vertebrate organism, such as man or other mammal, birds, or fish. The preparation comprises cells, derived from the tumor, that contain an exogenous gene (e.g., one introduced by insertion of genetic material from outside the cell, for example, by transfection and selection of a stably transfected cell line) which encodes an immunopotentiating interleukin. In a preferred embodiment, the interleukin is interleukin 2. Although applicants are not bound by theory, evidence suggests that the interleukin 2 gene confers upon the cell the ability to induce a specific immune response, probably mediated primarily by T lymphocytes.

In a related embodiment, the invention also provides a composition for enhancing non-specific resistance to a tumor, that embodiment including a preparation of cells containing an exogenous gene encoding interferon, preferably, interferon  $\gamma$ .

Although it is not necessary for practice of the invention, various gene combinations may produce greatly advantageous or even synergistic effects on potentiation of immunity to the tumor. Therefore, the invention also provides immunotherapeutic cells that may contain more than one exogenous gene. For example, the cells may contain either an interleukin gene or an interferon gene or both and another gene encoding an yet another immunopotentiating polypeptide.

The immunopotentiating polypeptide may be defined as a polypeptide that enhances responsiveness of a host immune system to a tumor present in the animal. At least two categories of immunopotentiating genes can be used in practice of the invention. A first category, "immunopotentiating antigens," includes genes encoding a

variety of antigens alien to the animal, such as virus envelope proteins, bacterial cell antigens, and other antigens, capable of eliciting an immune response in an animal immunized with a cell expressing such antigen.

5 That category of genes is referred to here as "immunopotentiating antigens." Preferred species among the category include, for example, viral envelope proteins, e.g., the influenza hemagglutinin (HA) gene, the influenza neuraminidase gene, vaccinia genes, or vesicular  
10 stomatitis virus genes. Suitable bacterial antigens include, for example, the 65kDa antigen of *M. tuberculosis* and other bacterial antigens. Other immunopotentiating genes may include those encoding histocompatibility antigens allogenic to the host and other alloantigens.

15 A second category of immunopotentiating genes includes genes encoding proteins that may not be immunogenic to the host but nevertheless potentiate immunity by activating or enhancing the activity of cells of the immune system, such as T lymphocytes, natural  
20 killer cells, or lymphokine activated killer cells. Included in this category of immunopotentiating genes are those encoding a number of the lymphokines classified as "interleukins," and in particular, interleukin-2, -4, -5, -6, -1, or -3. Also included are in this category, although  
25 not necessarily working according to the same mechanism, are proteins known as interferons, and in particular interferon gamma.

The genes may be obtained by techniques known in molecular biology and introduced to a selected target cell  
30 (which is usually derived from the tumor) by transfection or by transformation with a vector suitable for use with the particular target cell. Particular techniques described by Sambrook, et al., Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor  
35 Press, 1989 (incorporated herein by reference) may facilitate practice of certain aspects of the invention.

In one embodiment of the invention, one may employ a population of selected target cells containing at least one gene encoding an immunopotentiating antigen and a second immunopotentiating gene selected from the second category of immunopotentiating genes discussed above.

The invention also includes methods for potentiating the immune response of a vertebrate organism to a tumor by introducing into the organism a viable preparation of the cells described here. In a preferred embodiment, the cells are administered after reduction of tumor burden, e.g., by surgical resection, irradiation, or other appropriate techniques.

Of course, in many situations, it may be desirable to selectively ablate or destroy the administered cells once they have achieved the desired function. Therefore, in another major embodiment of the invention, the cells containing the immunopotentiating gene also contain a "lethal gene" that allows one to selectively kill the cell containing it should it become desirable to do so. In a preferred embodiment, the lethal gene will be coupled to a specifically inducible promoter, so that by treatment with the inducer, one can specifically promote transcription of the mRNA encoding the "lethal protein," which, in turn, is capable of directly or indirectly killing the host cell, for example, by inhibition of intracellular metabolism.

Therefore, according to this embodiment the invention provides a cell, which comprises a first exogenous gene encoding a selected polypeptide and a second exogenous gene ("lethal gene") including a promoter, preferably a selectively inducible promoter, in operative linkage to DNA encoding a second selected polypeptide. The second selected polypeptide, a "lethal polypeptide," is capable of killing the cell, for example, upon induction of a selectively inducible promoter or upon addition of a substrate for the lethal polypeptide, which substrate is converted by the lethal polypeptide to a molecule that is



toxic for the cell. By the term "operative linkage," it is meant that the promoter is located in a position relative to that of the DNA encoding the lethal polypeptide that allows the promoter to effectively direct transcription of the structural gene for the lethal polypeptide.

In a more specific embodiment directed to cancer immunotherapy, the invention provides a composition for potentiating the immune response to a tumor, the composition comprising a cell derived from the tumor, which cell contains a first exogenous gene encoding an immunopotentiating polypeptide and a second exogenous gene including a promoter in operative linkage to DNA encoding a polypeptide capable of killing the cell. Preferably, the promoter will comprise a specifically inducible promoter and the lethal polypeptide kills the cell upon induction of the promoter.

Although any of a number of suitable promoters, such as the SV40, cytomegalovirus, or actin promoters may find utility in practice of this aspect of the invention, the inducible 6-16 interferon  $\alpha/\beta$  promoter described below is particularly advantageous. Alternatively, when a gene encoding a polypeptide which requires addition of an exogenous substrate for intracellular toxicity is used (such as the Herpes simplex thymidine kinase that requires gancyclovir), a constitutive promoter such as the Herpes simplex promoter may be employed.

A number of suitable lethal genes may be used. Suitable examples include the Herpes simplex virus thymidine kinase gene and the gene encoding Diphtheria toxin A chain.

The cells containing immunopotentiating and lethal genes may also include yet a third exogenous gene, encoding a second immunopotentiating polypeptide. In a preferred embodiment, the third exogenous gene encodes an immunopotentiating antigen if the first immunopotentiating

polypeptide is a cytokine and encodes a cytokine if the first immunopotentiating polypeptide is an antigen.

The invention also includes methods for producing the novel cell derivatives. The invention includes, for  
5 example, a method for producing a cell for therapy of a selected disease comprising introducing a first exogenous gene encoding a selected polypeptide into a cell and introducing a second exogenous gene containing a promoter, preferably a selectively inducible promoter, in operative  
10 linkage with DNA encoding a polypeptide capable of killing the cell, into the cell to produce a cell having both the first and the second exogenous gene. Provision of a selectively inducible promoter allows one to kill the cell upon induction of the promoter. Cells, genes, and  
15 promoters, and lethal genes as described above are suitable for use with this aspect of the invention as well.

Of course, the invention also includes methods for use in gene therapy, and specifically, immunotherapy of  
20 cancer. An exemplary method for gene therapy of a vertebrate organism comprises introducing a first exogenous gene encoding a selected polypeptide into a tumor cell and introducing a second exogenous gene containing a promoter in operative linkage with DNA  
25 encoding a polypeptide capable of killing the cell into the cell to produce a cell having both the first and the second exogenous genes stably maintained in the cell; and administering the cell, preferably by injection into a the organism. In the preferred embodiment where the promoter  
30 is a selectively inducible promoter, the additional step of inducing the promoter to promote synthesis of the lethal polypeptide may be performed when warranted. Alternatively, when a constitutive promoter is used, e.g, in conjunction with the Herpes simplex thymidine kinase  
35 gene, administration of a second toxic agent, such as gancyclovir, may comprise an additional step of the

-9-

methods. Of course, the Herpes simplex thymidine kinase gene may also be placed under control of a selectively inducible promoter, in which case both induction of the promoter and administration of the second agent may occur.

5       The methods may be used for immunotherapy of a variety of tumors, including, for example, melanoma, breast cancer, sarcoma, colon cancer, and ovarian cancer.

10       These and other aspects of the invention will become more apparent from a description of particular embodiments when read in conjunction with the drawings.

#### FIGURE 1

15       Induction of CTLs after In Vivo Injection of CT26 cells and IL-2-Transfected CT26-IL-2<sup>+</sup> Cells. Cells ( $1 \times 10^6$ ) of either the CT26 or CT26-IL-2<sup>+</sup> lines were injected subcutaneously into the left flank of BALB/c mice. After 2 weeks, splenocytes were removed and cultured 5 days with mitomycin C-treated CT26 cells in the presence of IL-2. At the end of culture, live cells were mixed with <sup>51</sup>Cr-  
20       labeled CT26 targets at different effector to target ratios in a 4 hr <sup>51</sup>Cr release assay. (a) CTLs generated from CT26 vs. CT26-IL-2<sup>+</sup> cells. (b) Anti-CD4 and anti-CD8 blocking of CT26 lysis by splenocytes from mice immunized with CT26-IL-2<sup>+</sup> cells. (c) Anti-MHC class I and class II  
25       blocking of CT26 lysis by splenocytes from mice immunized with CT26-IL-21<sup>+</sup> cells.

#### FIGURE 2

30       Protective Immunity against CT26 Cells Induced by Injection of CT26-IL-2<sup>+</sup> Cells. BALB/c mice were injected subcutaneously in the left flank with  $1 \times 10^6$  CT26-IL-2<sup>+</sup> cells followed by subcutaneous challenge in the right flank with  $1 \times 10^5$  CT26 cells either 2 weeks or 4 weeks later. Also shown is CT26 growth without CT26-IL-2<sup>+</sup> pre-  
35       immunization. Tumor growth was assessed every week by palpation and measurement. The pooled results of 20 mice

-10-

per group are shown. Data is presented as a Kaplan-Meier plot.

**FIGURE 3**

5 Effects of T Cell Subset Depletion on the In Vivo Response  
to CT26-IL-2<sup>+</sup> and CT26-HA<sup>+</sup> Cells. BALB/c mice were  
depleted in vivo of either CD4<sup>+</sup>, CD8<sup>+</sup>, or both CD4<sup>+</sup> and  
CD8<sup>+</sup> T cells by intraperitoneal injection of purified  
10 anti-CD4 or anti-CD8. They were then injected  
subcutaneously in the left flank with 1 x 10<sup>6</sup> CT26-neo-IL-  
2<sup>+</sup> cells. Also shown is the growth in normal and CD4-  
depleted BALB/c mice of a CT26-HA<sup>+</sup> line rendered  
immunogenic by transfection with the influenza  
hemagglutinin gene. Tumor growth was measured every week  
15 and is presented as in Figure 2. Specific depletion of T  
cell subsets was documented as described in Experimental  
Procedures.

**FIGURE 4**

20 Fluorescence intensity distribution based on the binding  
of labeled goat anti-mouse immunoglobulin to SP1 or CT-26  
cells pre-treated with mouse anti-H2K<sup>k</sup> or H2K<sup>d</sup>,  
respectively.

**FIGURE 5**

25 SP1 cells were treated with 10 U/ml of IFN<sub>γ</sub> for 24 hours.  
The cells were washed and a sample was removed for FACS  
analysis. The cells were returned to in vitro culture  
without additional IFN<sub>γ</sub> and samples were removed for FACS  
30 analysis at 48, 72 and 96 hours. This figure shows the  
fluorescence intensity distribution based on binding of  
goat anti-mouse immunoglobulin to IFN<sub>γ</sub> treated SP1 cells  
pre-treated with mouse anti-H2K<sup>k</sup> antibody.

**FIGURE 6**

Three distinct clones of SP1 cells transfected with the murine IFN $\gamma$  gene were assessed for H2K<sup>t</sup> expression. The figure demonstrates the fluorescence intensity distribution based on the binding of goat anti-mouse immunoglobulin to cells pre-treated with mouse anti-H2K<sup>t</sup>.

**FIGURE 7**

A group of six nude mice were injected s.c. with  $1 \times 10^5$  6L cells. Two of the six resulting tumors were removed, cultured in vitro and assessed for IFN $\gamma$  production and H2K<sup>t</sup> expression. The individual tumors 6L-A and 6L-B were then injected into syngeneic mice and assessed for their tumorigenicity.

The following examples illustrate selected aspects of the invention but are not intended to limit it except as specified in the claims.

**EXAMPLE I**

Enhancement of Anti-Tumor Immunity by Immunization with Cells Transfected with an Exogenous Gene Encoding Interleukin 2.

The following example describes results obtained upon practice of one aspect of the invention--potentiation of an anti-tumor immune response by administering cells transfected with a gene encoding an exogenous immunopotentiating cytokine, in this case, interleukin 2.

30

**A. Experimental Procedures****1. Cells**

CT26 cells were obtained from M. Brattain (Brattain et al., 1980). The F10 subline of B16 melanoma cells (Fidler, 1975) was obtained from the NIH DCT tumor repository. RENCA is a murine renal cell carcinoma originally described by Murphy and Hausheky (1973). SS-5

-12-

is a spontaneous mammary adenocanthoma induced by methylcholanthrene in one of our laboratories (P.F.). YAC-1, an MHC NK target cell line (Kiessling et al., 1975), was kindly provided by J. Wagner, Johns Hopkins University.

## 2. Transfections

DNA was introduced into cells as a coprecipitate with calcium phosphate (Graham and van der Eb, 1973; Wigler et al., 1979). The CT26-IL-2<sup>+</sup> cell line was obtained by transfection with 5 µg of the plasmid vector pBCMG-neo-mIL-2, a bovine papilloma virus expression vector containing a murine IL-2 cDNA clone under the transcriptional control of a cytomegalovirus promoter with a rabbit β-globin intron, splice, and poly(A) addition signals; it also contains the Th5 neomycin-resistance gene (Karasuyama and Melchers, 1988; Karasuyama et al., 1989). Cells were exposed to the precipitate for 14-16 hr, washed once with Hanks' balanced salt solution without Ca<sup>2+</sup> or Mg<sup>2+</sup>, refed with Dulbecco's modified Eagle's medium with 10% FCS, and incubated at 37°C. Selection in G418 at 400 µg/ml was begun 48 hr after cells were exposed to precipitate. The CT26-neo-IL-2<sup>-</sup> line was produced by transfection of CT26 cells with a plasmid derived from pBCMG-neo-IL-2 by removing the cytomegalovirus early promoter, rabbit β-globin intron, and mIL-2 sequences. The hemagglutinin-expressing CT26-HA<sup>+</sup> cell line (described in Fearon et al., 1988) was produced by cotransfection of CT26 with 5 µg of the plasmid vector pBV1-MTHA and pSV2-neo followed by G418 selection. FACS 3, clone 5 was used in the studies here. The B16-IL-2<sup>+</sup> transfectant was generated similarly to the CT26-IL-2<sup>+</sup> transfectant but was cloned by limiting dilution after G418 selection.

-13-

### 3. IL-2 Assays

Supernatants of transfected cells were assayed for IL-2 as previously described (Janis et al., 1989) by transferring dilutions to 96-well microtiter plates containing 3000 CTLL-2 cells per well. After 24 hr, [<sup>3</sup>H]thymidine was added for 12 hr, after which incorporation was assessed with a PHD cell harvester. Units per milliliter IL-2 was calculated as the reciprocal of the supernatant dilution giving half-maximal proliferation of CTLL-2.

### 4. CTL Assays

For CTL assays, spleens were removed from BALB/c mice 2 weeks after subcutaneous injection in the left flank of 1 x 10<sup>6</sup> CT26 or CT26-IL-2<sup>+</sup> cells. Mitomycin C treatment of CT26 cells was performed by incubating them in 50 µg/ml mitomycin C for 45 min at 37°C followed by three washes with RPMI-10 FCS. In vitro stimulations were performed in 24-well plates for 5 days with 2 x 10<sup>5</sup> CT26 stimulators and 6 x 10<sup>6</sup> splenocyte responders per well plus 20-50 U/ml recombinant murine IL-2. <sup>51</sup>Cr release assays were performed by mixing various numbers of effector cells with 5000 <sup>51</sup>Cr-labeled targets per well in 96-well V-bottom plates. After 4 hr at 37°C, 100 µl per well was removed and counted in a gamma counter. Percent specific lysis ( $[(\text{cpm}_{\text{exp}} - \text{cpm}_{\text{min}})/(\text{cpm}_{\text{max}} - \text{cpm}_{\text{min}})] \times 100$ ) is plotted on the y-axis for various effector to target ratios. CTL assays for the B16 melanoma system were performed in a similar fashion, except that incubations with <sup>51</sup>Cr-labeled B16 targets were performed for 8 hr. For antibody blocking, a 1:100 dilution of ammonium sulfate-purified preparations of the following antibodies were added to microwells at the start of <sup>51</sup>Cr release assays: GK1.5, monoclonal antibody (MAb) to CD4 (Dialynis et al., 1983; 2.43, MAb to CD8.2 (Sarmiento et al., 1980); M5-114, MAb to I-A<sup>d</sup> + I-E<sup>d</sup> (Battacharaya et al., 1981); 28-14-8, MAb to I<sup>d</sup> (Ozato

et al., 1980); 35-1-2, MAb to K<sup>d</sup> + D<sup>d</sup> (Ozato et al., 1982). All blocking antibodies were carefully titered such that the concentrations used were 5-10 times the minimal concentration that yielded saturation binding to splenic lymphocytes as assayed by flow cytometric analysis of serially diluted antibody. Prior to use, blocking specificity was assessed as follows: anti-CD4 and anti-MHC class II antibodies inhibited in vitro secondary PPD-proliferative responses by more than 80% and allo-CTL lysis (B6 anti-BALB/c) by more than 5%; anti-CD8 and anti-MHC class I antibodies inhibited secondary PPD-proliferative responses by more than 5% and allo-CTL lysis by more than 80%.

15                    5.    In Vivo Antibody Depletions

In vivo antibody depletions were started 1-2 days prior to injection of the tumor. MAb GK1.5 was used for CD4 depletions and MAb 2.43 was used for CD8 depletions. Ammonium sulfate-purified ascites fluid (titered at >1:2000 by staining of thymocytes on the FACS) was injected intraperitoneally (0.1 ml per mouse) every other day for the first 3 weeks and then once per week afterward. Depletion of T cell subsets was assessed on the day of tumor injections, 3 weeks, and 5 weeks after tumor injection by flow cytometric analysis of lymph node cells stained with 2.43 or GK1.5, followed by fluorescein isothiocyanate-labeled goat antibody to rat IgG. For each time point of analysis, >99% depletion of the appropriate subset was achieved with normal levels of the opposite subset present (in the case of the single depletions).

B.    Results

1.    Production of IL-2 Transfected CT26 Cells

The N-nitroso-N-methylurethane-induced murine colon tumor line CT26 chosen for study is poorly immunogenic; a small number of cells ( $1 \times 10^3$ - $1 \times 10^4$ ) injected into



-15-

syngeneic (BALB/c) mice cause a lethal tumor and do not induce detectable tumor-specific CTLs (Fearon et al., 1988). CT26 cells were transfected with a bovine papilloma virus (BPV) vector containing a neomycin-resistance gene and a murine IL-2 CDNA. Transfectants were selected in the neomycin analog G418, and a G418-resistant line (CT26-IL-2<sup>+</sup>) prepared from more than 50 pooled clones of approximately equal size was chosen for further study.

To assay for IL-2 production, the CT26-IL-2<sup>+</sup> line was plated at 5 x 10<sup>4</sup> cells per well in a 24-well plate, and after 3 days, supernatants (1.5 ml per well) were removed and transferred in serial dilutions to the IL-2-dependent CTLL-2 cell line. They were found to contain 40 U of IL-2 activity (1 U defined as induction of 50% maximal CTLL stimulation in a 24 hr assay), indicating that CT26-IL-2<sup>+</sup> cells secreted significant quantities of IL-2. No detectable IL-2 activity was found in supernatants from either the parental CT26 cells or CT26 cells transfected with the BCMG vector that had the IL-2 cDNA insert cut out.

## 2. CTL Generation Induced by CT26-IL-2<sup>+</sup> Cells

Subcutaneous injection of CT26 cells has been shown previously to elicit little if any detectable systemic CTL activity, even after a secondary in vitro stimulation in the presence of IL-2 (Fearon et al., 1988). However, after subcutaneous injection of the IL-2-producing CT26-IL-2<sup>+</sup> cells, significant anti-CT26 CTL activity was detected in the spleen after secondary in vitro stimulation (Figure 1a). The majority of in vitro CTL activity was blocked by antibodies to CD8 and to MHC class I, but not by antibodies to CD4 or to MHC class II (Figures 1b and 1c). This suggested that CT26-IL-2<sup>+</sup> cells indeed activated endogenous, MHC class I-restricted CD8<sup>+</sup> CTLs. Virtually no cytotoxicity was observed against another

BALB/c-derived tumor target (SS-5) or the MHC class I<sup>-</sup> NK target, YAC-1 (<5% specific lysis at an effector to target ratio of 100:1). These results demonstrate that most of the effector cells induced by CT26-IL-2<sup>+</sup> immunization were antigen- and MHC class I-specific and that NK and LAK cells represent a minority of the activity measured in vitro.

3. In Vivo Immune Response Induced by CT26-IL-2<sup>+</sup> Cells

We next sought to determine whether the CTL response elicited by CT26-IL-2<sup>+</sup> cells correlated with in vivo immunity. Following even the largest subcutaneous injection of up to  $1 \times 10^6$  CT26-IL-2<sup>+</sup> cells, no tumors were seen up to 8 weeks after injection. In contrast, tumors were present in all animals following injection of the parental CT26 cells by 2 weeks (Table 1). Thus, BALB/c mice were capable of rejecting 3-4 orders of magnitude greater numbers of CT26-IL-2<sup>+</sup> cells than the parental CT26 cells. To demonstrate that rejection of CT26-IL-2<sup>+</sup> cells is due to activation of CT26-specific effector cells by the locally produced IL-2, CT26 cells were transfected with the entire BPV vector sequences alone with the IL-2 insert removed. These transfectants (CT26-neo-IL-2<sup>-</sup>), which were confirmed not to secrete IL-2 by CTL functional assay, produced tumors in BALB/c mice at a rate indistinguishable from the nontransfected CT26 cells (Table 1). In addition, we observed that injection of  $1 \times 10^6$  CT26-IL-2<sup>+</sup> cells on the left flank did not inhibit the growth of  $1 \times 10^5$  CT26 cells on the opposite flank. Thus, the effect of IL-2 production by CT26-IL-2<sup>+</sup> cells appeared initially to stimulate a local, rather than systemic, immune response.

Given the result that immunization with CT26-IL-2<sup>+</sup> cells induced systemic CTLs after two weeks, as measured in vitro, we sought to determine whether this correlated

-17-

with an *in vivo* antitumor response against the parental CT26 cells. Indeed, injection of CT26-IL-2<sup>+</sup> cells completely protected mice against a challenge with 1 x 10<sup>5</sup> CT26 cells 2 weeks later (Figure 2). This protection was not long-lived, as roughly 50% of mice challenged 4 weeks after immunization developed tumors (Figure 2). The protection was tumor specific in that other BALB,c tumors (SS-5, RENCA) grew normally when injected 2 weeks after CT26-IL-2<sup>+</sup> immunization (data not shown).

#### 4. CT26-IL-2<sup>+</sup> Cells Bypass CD4<sup>+</sup> T Helper Function

Because most T cell helper function is performed by the CD4<sup>+</sup> subset of T lymphocytes and most MHC-restricted CTL function is performed by the CD4<sup>+</sup> subset, we next investigated the effect of selective *in vivo* depletion of these subsets on the rejection of CT26-IL-2<sup>+</sup> cells. Figure 3 shows that mice depleted of CD4<sup>+</sup> T cells were fully capable of rejecting CT26-IL-2<sup>+</sup> cells. Previous studies have shown that CT26 cells could be rendered immunogenic by transfection with a foreign gene such as influenza hemagglutinin (CT26-HA<sup>+</sup>; Fearon et al., 1988). The "failure of help" hypothesis suggested that the enhanced immune response induced by CT26-HA<sup>+</sup> cells might be mediated, at least in part, by CD4<sup>+</sup> helper T cells responding to MHC class II-restricted epitopes on the exogenously introduced hemagglutinin gene product. In support of this hypothesis is the observation that CT26-HA<sup>+</sup> cells failed to be rejected by CD4-depleted mice (Figure 3). Taken together, these results support the concept that CT26-IL-2<sup>+</sup> immunization effectively bypasses Th function in the generation of an antitumor CTL response.

#### 5. CD8<sup>+</sup> Cells Are Required In Vivo For Tumor Rejection

Mice depleted of CD4<sup>+</sup>8<sup>+</sup> cells were incapable of rejecting CT26-IL-2<sup>+</sup> cells, indicating that this T cell subset is involved in the antitumor response in vivo (Figure 3). It is noteworthy, however, that there was a significant lag in the tumor's growth kinetics relative to the kinetics of CT26 growth in normal BALB/c mice. While this partial response could be due to the action of residual CD4<sup>+</sup>8<sup>+</sup> cells, none were detected in lymph nodes by flow cytometric analysis either at the time of initial tumor injection or during the course of in vivo antibody treatment. A similar delay in tumor growth was seen in the presence of both CD4 and CD8 depletion. It is therefore likely that an IL-2-responsive, CD4<sup>+</sup>8<sup>+</sup> effector cell population was additionally involved in the in vivo antitumor response. Three IL-2-responsive candidates are NK cells, LAK cells, and T cells bearing the  $\gamma\delta$  T cell receptor (reviewed in Pardoll et al., 1987; Brenner et al., 1988; Raulet, 1989). As most of the cells in these populations are CD4<sup>+</sup>8<sup>+</sup>, they would still be present in CD8-depleted mice. Other potential effector cells involved in the antitumor response, based on histologic analysis of regressing tumors, include macrophages and mast cells (data not shown).

#### 6. CTL Generation and In Vivo Immunity Induced by IL-2-Producing Melanoma Cells

To assess whether the induction of systemic immunity was a general feature of tumors engineered to secrete IL-2, we transfected a second poorly immunogenic tumor, B16 melanoma, with the BCMG-IL-2 vector. The B16 melanoma is a highly aggressive melanocyte tumor of C57BL/6 origin. After transfection, G418-resistant clones were chosen (B16-IL-2<sup>+</sup>) that produced quantities of bioactive IL-2 equal to or greater than cells, when assayed under

-19-

identical conditions on CTLL cells. Table 2 shows the results of a representative clone. While small numbers of B16 melanoma cells caused tumors in syngeneic C57BL/6 mice, B16-IL-2<sup>+</sup> cells were completely rejected. As with the CT26 tumor, greater CTL activity was generated against the parental B16 tumor in mice injected with B16-IL-2<sup>+</sup> cells. C57BL/6 mice injected with B16-IL-2<sup>+</sup> cells developed protective immunity in vivo against challenge with B16 cells in a dose-dependent manner. The protection was not as great as seen in the CT26 system, as roughly half the mice immunized with  $1 \times 10^6$  B16-IL-2<sup>+</sup> cells did eventually develop tumors after challenge with  $1 \times 10^5$  parental B16 cells (Table 2).

It is noteworthy that MHC class I expression on B16 melanoma cells is extremely low - roughly 5- to 10-fold lower than on CT26 cells (data not shown). This may account for the apparently lower efficiency of protection against challenge with parental tumor in the B16 than in the CT26 system, although the faster growth rate of B16 melanoma cells may be an additional contributing factor.

In addition to the colon tumor and melanoma data presented here, similar results have been recently observed with an IL-2-transfected murine CBA-SP1 sarcoma and the rat Dunning prostate carcinoma (data not shown). The fact that similar effects were seen with two tumors of widely differing cellular origins suggests that the principles outlined here may be generalizable to a variety of cancers. In other studies, however, a spontaneous mammary adenocarcinoma derived from CBA mice was transfected with the IL-2 gene and used to immunize CBA mice against growth of the parental tumor. In those studies, the IL-2<sup>+</sup> transfectant did not induce a significant immunity against the parental tumor.

We have also prepared a number of additional transfectants expressing HA and/or secreting IL-2 or IFN-

γ. It is believed that the dually transfected cells should improve the immunogenic status of the tumor.

#### EXAMPLE II

5     Production of Tumor Cells Transfected with an Exogenous Gene Encoding Gamma Interferon and Demonstration of Enhanced Non-Specific Tumor Resistance

10     The following example describes yet another aspect of the invention, transfection of cells with a gene encoding a cytokine capable of non-specifically enhancing tumor resistance, probably through a mechanism dependent on cells other than T lymphocytes. In the following example, the non-specific enhancement of tumor resistance was produced by injecting mice with a tumor cell transfected with the gene encoding gamma interferon.

15     More specifically, SP1 murine adenocarcinoma cells transfected with the murine IFN<sub>γ</sub> gene expressed IFN<sub>γ</sub> (SP1/IFN<sub>γ</sub>), failed to grow in syngeneic hosts, and grew in nude mice. The rejection of SP1/IFN<sub>γ</sub> cells was related to the amount of IFN<sub>γ</sub> produced and appeared to be mediated primarily by nonspecific cellular mechanisms, though some role for T cells in the afferent arm of this response is possible. SP1 are H2K<sup>b</sup> negative but express class I antigens when producing IFN<sub>γ</sub>. However, class I MHC expression, while likely necessary, was insufficient in itself to prevent tumor growth since secretion of >64 U/ml IFN<sub>γ</sub> was needed to inhibit tumorigenicity while only 8 U/ml IFN<sub>γ</sub> could induce class I antigens. Similar results were obtained with the murine colon carcinoma CT-26, a tumor that constitutively expresses class I MHC antigens, further supporting the contention that Class I MHC expression is not essential for the rejection response induced by IFN<sub>γ</sub>. The failure of SP1/IFN<sub>γ</sub> cells to protect against a challenge with parent SP1 cells argues that factors other than IFN<sub>γ</sub> production or class I MHC

20  
25  
30  
35

-21-

expression are needed to induce a protective response against weakly or non-immunogenic tumor cells.

A. Materials and Methods

5

1. Mice.

CBA and BALB/c female mice between 6 and 8 weeks of age were obtained from the Frederick Animal Facility of the National Cancer Institute.

10

2. Tumors.

The origin of the SP1 spontaneous mammary adenocarcinoma has been described previously (Frost et al., 1987). SP1 grows readily in syngeneic mice after s.c. inoculation and is poorly, if at all, immunogenic and expresses little if any class I MHC antigen. The CT-26 colon adenocarcinoma was obtained from M. Brattain (Brattain et al., 1980). MDW1 is an immunogenic variant of the MDAY-D2 lymphoma (Kerbel, 1979).

15

20

3. Culture Conditions.

Cells were cultured in GIBCO RPMI 1640 medium supplemented with 50,000 U of penicillin and streptomycin, 150 mg of L-glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 375 mg of sodium bicarbonate, and 10% fetal calf serum (Irvine Scientific, Santa Ana, CA). All tumors were tested periodically for the presence of *Mycoplasma* using the Gen Probe RNA hybridization method (Gen Probe, San Diego, CA); contamination with *Mycoplasma* was never observed. In addition, the cells were tested for murine antibody production (Microbiological Associates, Bethesda, MD) and found to be free of 13 murine pathogenic viruses.

25

30

4. Antisera.

35

The monoclonal antibodies used in these studies were as follows: Murine anti-D<sup>k</sup>/K<sup>d</sup> (clone 15-5-5S) was kindly

provided by Dr. B.E. Elliott (Queen's University, Kingston, Ontario, Canada). Murine anti-K<sup>d</sup> (clone 16-1-11N) was purchased from the Litton Company (Charles, SC). Murine anti-K<sup>k</sup>/D<sup>k</sup> Ab (clone H 100-27/55), anti-I-A<sup>k</sup> (clone 14V18), and anti-D<sup>k</sup> were purchased from the Cedarlane Company (Ontario, Canada). Murine anti-I-A<sup>d</sup> antisera was obtained from Becton Dickinson (San Jose, CA).

5. In Vitro IFN<sub>γ</sub> Treatment.

Recombinant murine IFN<sub>γ</sub> was obtained from Genentech, Inc. (San Francisco, CA). 3 x 10<sup>5</sup> tumor cells were plated in five 10-cm plastic dishes and incubated with 1-100 U/ml of IFN<sub>γ</sub> for 4 days. On the fourth day, IFN<sub>γ</sub>-treated cells were aliquoted and reincubated with 1-100 U/ml of IFN<sub>γ</sub> for an additional 3 d. The cells were then analyzed for the expression of class I or class II MHC antigens.

6. Flow Cytofluorometry.

Quantitative analysis of MHC expression on cell surfaces were performed using FACS. Cells were scraped from tissue culture plates using a rubber policeman and incubated with murine anti D<sup>k</sup>, D<sup>d</sup> or IA<sup>k</sup> or IA<sup>d</sup> antibodies for 30 min at 4°C. After being washed, the cells were incubated with FITC-conjugated goat anti-mouse IgG antibodies at 4°C for 30 min, fixed with 1% paraformaldehyde and examined within one week using cytofluorometry.

7. Transfection of Cells With DNA.

DNA was introduced into SP1 or CT-26 cells either as a coprecipitate with calcium phosphate (Graham and van der Eb, 1973) or via use of the lipofectin reagent (Felgner et al., 1987). The SP1-neo and CT-26/neο cell lines were obtained by transfection with a mixture of 1 ug of the plasmid vector pSV2neo with 1 ug of mouse liver DNA. The SP1-IFN<sub>γ</sub> lines were obtained by transfecting SP1 cells



with 1 ug of pSV2neo and 10 ug of pGEM3-SVMu IFN $\gamma$ . The latter is a pGEM vector containing an SV40 promoter and the murine IFN $\gamma$  gene and was kindly provided by Dr. R. Suzuki of the Department of Immunology at the M.D.

5 Anderson Cancer Center. After selection in geneticin, the colonies from individual plates were pooled and assessed for IFN $\gamma$  production.

8. Anti-Viral Assay for Interferon Activity.

10 The method of Mory et al., was used (Mory et al., 1981) to assay IFN activity. This assay relies on the inhibition of vesicular stomatitis virus growth in murine LTK cells. Control wells were treated with recombinant IFN $\gamma$  or medium alone. Supernatants from the experimental  
15 cell cultures were added at doubling dilutions and their ability to inhibit VSV viral growth assessed by determining how many wells contained viable LTK cells.

9. Assessment of Cell-Mediated Cytotoxicity.

20 Cytotoxicity was measured using the previously described <sup>111</sup>In-release assay (Wiltrout et al., 1978).

10. Separation of Spleen Cells.

Nylon wool adherent (NWA) and nonadherent cells  
25 (NNA) were obtained by the method described in Julius et al., (1973).

B. Results

Prior to the transfection studies, we ascertained  
30 whether the SP1 cells would express MHC class I antigens in response to IFN $\gamma$ . In addition, we determined if the addition of exogenous IFN $\gamma$  had a cytostatic effect on either the SP1 or CT-26 cell lines. One to 1000 U of IFN $\gamma$  were added to semiconfluent cultures of SP1 or CT-26 cells  
35 for four days. Class I MHC expression was then measured and cell viability and replication was measured each day

for five days and compared to non-IFN $\gamma$ -treated cells. CP1 cells grew normally in as much as 100 U/ml IFN $\gamma$ ; but the growth of CT-26 cells was inhibited by 17% in 1000 U/ml IFN $\gamma$ . The growth rates of SP1 cells transfected and  
5 secreting 256 U/ml IFN $\gamma$  (6L) were the same as that of the parent SP1 cells. Similarly, CT-26 cells producing 64 U/ml IFN $\gamma$  had the same growth rate as parent CT-26 cells.

Figure 4 demonstrates the expression of H2K<sup>k</sup> in Sp1 cells as well as H2K<sup>d</sup> in CT-26 cells as assessed by FACS  
10 using specific murine monoclonal antibodies. Fewer than 6-7% Sp1 cells expressed H2K<sup>k</sup>, but all expressed IA<sup>k</sup> (data not shown). In contrast CT-26 cells expressed both class I and II MHC antigens (Class II data not shown). After treatment of SP1 cells with exogenous IFN $\gamma$ , the expression  
15 of class II antigens did not change but a dramatic shift of the FACS curve to the right was readily detectable with anti K<sup>k</sup> antibodies. IFN $\gamma$  treatment had no effect on the constitutively expressed class I or II antigens of CT-26 cells.

20 We next determined the duration of the effect of exogenous IFN $\gamma$  on class I expression by SP1 cells. These experiments served as a basis for the use of transfection with the IFN $\gamma$  gene as a means for providing sustained production of IFN $\gamma$  and expression of class I MHC antigens.  
25 SP1 cells were treated with 100 u/ml of IFN $\gamma$  for three days. The cells were washed and either assessed for H2K<sup>k</sup> expression or recultured. This was repeated at 24, 48, 72 and 96 hours after the removal of IFN $\gamma$  from the medium. Figure 5 shows that the H2K<sup>k</sup> expression returned to  
30 baseline levels within 48 hours after the removal of IFN $\gamma$  from the medium. It is reasonable to assume that a similar reduction in H2 expression would occur if IFN $\gamma$ -treated cells were injected in vivo.

1. Effect of IFN<sub>γ</sub> Transfection on Class I MHC Expression.

After transfection, both the SP1 and CT-26 lines were tested for the secretion of IFN<sub>γ</sub>. Three SP1-IFN<sub>γ</sub> cell populations--1C, 3C and 6L-- were selected for study based on the amount of IFN<sub>γ</sub> secreted. The results are shown in Table 3. The CT-26/IFN<sub>γ</sub> cells were similarly shown to produce IFN<sub>γ</sub>, but to a lesser degree than the SP1/IFN<sub>γ</sub> cells (8 U/ml vs 256 U/ml). Of interest is the demonstration in all three SP1/IFN<sub>γ</sub> cell lines of an equivalent increase in MHC class I expression (Figure 6, the results for the CT-26 cells are shown below)). In addition, all three lines expressed class II MHC antigens (IA<sup>k</sup>) at a level comparable to the nontransfected parent cells (data not shown). SP1 cells transfected with the neo<sup>R</sup> gene alone did not express class I MHC antigens.

2. Effect of IFN<sub>γ</sub> Gene Transfection on Tumor Growth In Vivo.

SP1/IFN<sub>γ</sub> were injected s.c. into CBA mice. Table 3 summarizes the data from several such experiments. These cell lines were selected because they all demonstrated a significant level of H2K<sup>k</sup> expression (Figure 6), when compared to five additional populations that were screened and found not to express H2K<sup>k</sup> and were thus presumed not to be producing IFN<sub>γ</sub>. 1C and 3C grew at challenge doses of  $1 \times 10^5$ . In contrast, 6L cells failed to grow in 14 of 15 animals injected with  $1 \times 10^6$  cells; the parent SP1 cell line grew when as few as  $10^3$  cells were injected s.c. Immunogenicity correlated with the level of IFN<sub>γ</sub> production rather than H2K<sup>k</sup> expression (Table 3 and Figure 6) since all three lines expressed equivalent levels of MHC antigen but only the 1C and 3C cell lines grew vigorously. 6L cells did grow in nude mice. SP1 cells transfected with the neo<sup>R</sup> gene alone grew in a manner

identical to nontransfected SP1 cells (Kerbel et al., 1987).

### 3. Nonspecific Cytotoxic Response to 6L.

5        Table 4 shows that viable 6L cells induced a cytotoxic response reactive with itself and the parent SP1 tumors. This response was mediated by nylon wool adherent (NWA) spleen cells but not by nylon wool non-adherent (NWNNA) cells. The cytotoxicity of the NWA spleen cells  
10        against both SP1 and 6L was always greater than that produced by the whole spleen cell population. In addition, NWA cells were reactive (though to a lesser degree) against the unrelated MDW1 tumor cell line. These results implied that a non-specific mechanism was  
15        responsible for the rejection of 6L cells. This view was supported by our failure to diminish the splenic cytotoxic response after depletion of immune spleen cells with anti-CD4, anti-CD8 and anti-CD3 antibodies plus complement (data not shown).

20        To further assess the nonspecific nature of the rejection of 6L cells,  $1 \times 10^6$  6L cells were mixed with  $1 \times 10^5$  SP1 cells and injected together into the left flank of CBA mice. Five of 10 mice developed tumors at 5 weeks. Syngeneic CBA mice injected with  $1 \times 10^4$  SP1 cells develop  
25        tumors within 2 weeks. The five tumors that grew were removed and placed in tissue culture, allowed to grow for two days, and then exposed to geneticin. In all cases, the cells placed in culture failed to grow in the presence of geneticin, indicating that these were parent SP1 cells,  
30        which are sensitive to 500 ug/ml geneticin (6L cells carry the neo<sup>R</sup> originally used in the co-transfection with the IFN <sub>$\gamma$</sub>  gene). The fact that the growth of 6L mixed with SP1 cells was markedly impaired argues that nonspecific mechanisms induced by the 6L cells were responsible in  
35        part for its failure to grow in vivo.

-27-

This conclusion was indirectly supported by experiments in which immunization of CBA mice with 6L failed to protect animals against a significant challenge with parental SP1 cells (Table 5). Fifty percent of immunized animals were protected against a challenge with  $1 \times 10^4$  cells injected in the opposite flank. However, as few as  $5 \times 10^4$  SP1 cells induced tumors in 100% of the immunized animals. In addition, the injection of animals simultaneously in the left flank with 6L and right flank with SP1 had no effect on the growth of the SP1 cells, though the 6L cells failed to grow.

#### 4. Role of Class I MHC Antigen Expression.

It remained to be determined whether the effect of  $\text{IFN}_\gamma$  production was strictly due to its enhancement of class I MHC expression or if  $\text{IFN}_\gamma$  produced locally had other effects on the host. 6L cells were injected into 6 nude mice and produced tumors. Two of these tumors were removed (6L-A and 6L-B) and recultured. These cell lines were then assessed for  $\text{IFN}_\gamma$  production and H2 expression. Figure 7 shows that 6L-A and 6L-B had equivalent expression of  $\text{H2K}^k$  even though 6L-B produced 64x more  $\text{IFN}_\gamma$ . The reinjection of 6L-A and 6L-B into groups of six CBA mice demonstrated that low  $\text{IFN}_\gamma$ -producing 6L-A cells grew in all animals despite expressing  $\text{H2K}^k$ . In contrast, high  $\text{IFN}_\gamma$  producing 6L-B cells failed to grow.

#### 5. The CT-26 Tumor.

We next determined the effect of  $\text{IFN}_\gamma$  expression of murine tumor cells that constitutively express class I MHC antigens. CT-26 murine adenocarcinoma cells were transfected with the pGEM-SVMu- $\text{IFN}_\gamma$  plasmid and the original pool of transfected cells was found to secrete 8 U/ml of  $\text{IFN}_\gamma$ . Table 6 shows that at doses of  $1 \times 10^5$  s.c. or  $5 \times 10^4$  i.v., CT-26/ $\text{IFN}_\gamma$  cells eventually grew, but considerably more slowly than parent CT-26 cells.

The failure of CT-26/IFN $\gamma$  cells to grow in vivo was directly related to their IFN $\gamma$  production and could not be explained by any direct cytopathic effect of IFN $\gamma$  because 8 U/ml IFN $\gamma$  had no effect on CT-26 growth in vitro. In addition to reduced growth in vivo, CT-26/IFN $\gamma$  induced a cytotoxic response detectable in the spleens of immunized mice (data not shown). Parent CT-26 cells have never been shown to engender any detectable cytotoxic response.

### 10 EXAMPLE III

Production of Immunopotentiating Tumor Cells  
Co-Transfected With an Exogenous Gene Encoding a  
Selected Immunopotentiating Polypeptide and a  
15 Second Exogenous Gene Encoding a Polypeptide Capable  
of Ablating the Host Cell on Specific Induction.

The following example describes yet another aspect of the present invention, the production of an immunogenic tumor cell that may be selectively ablated in circumstances when it becomes desirable to do so. In this aspect of the invention, cells are transfected with a selected gene encoding a desired polypeptide, e.g., the immunopotentiating polypeptide, and a second gene that encodes a polypeptide capable of ablating (killing) the host cell. The second gene, referred to as the "ablation gene" or the "lethal gene," is preferably under the control of a specifically inducible promoter. Thus, when it is desired to eliminate some or all of the transfected cells from the host (for example, at about 14 days after administration of the transfected tumor cells), one may initiate such elimination by directly or indirectly activating the inducible promoter to stimulate the synthesis of the lethal polypeptide, for example, by administering to the host an amount of the inducer sufficient to initiate transcription of the polypeptide. Specific examples of embodiments of this aspect of the invention are described below.

A. Selection and transfection of genes encoding immunopotentiating polypeptides

Selection of the most advantageous immunopotentiating polypeptide for use in a given situation will likely depend on a number of parameters, including, for example, tumor type, location, and immune status of the patient. Genes encoding such polypeptides are either currently available, or may be obtained using present techniques in molecular biology, for example, by using techniques described by Sambrook et al., in Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, 1989 (which is incorporated herein by reference). Transfection of the target cell type may generally be carried out essentially according to the protocol set forth in example I above. In the examples discussed more specifically below, the genes selected for transfection are those encoding the immunopotentiating polypeptides, IL-2, interferon gamma, and influenza hemagglutinin.

B. Selection and transfection with a specifically inducible lethal gene system.

1. Selection of a suitable promoter

Although a number of promoters, e.g., cytomegalovirus, SV40, or actin and the like could conceivably be used in accordance with the invention, we have selected the interferon-inducible 6-16 promoter for use in a preferred embodiment. Use of this promoter has a number of advantages because the levels of IFN in normal blood are very low. Therefore, the injection of IFN can trigger this gene in a very specific manner.

The human IFN-regulated gene 6-16 is tightly controlled by IFN  $\alpha/\beta$ . The 0.9 Kb cDNA of 6-16 encodes for a 13 kilodalton protein which contains a signal peptide sequence at the amino terminal end; however, neither the function or the subcellular localization of the protein (secreted or membrane bound) has been defined

(Kelly, 1986). Untreated cells have no detectable levels of 6-16 mRNA in their cytoplasm or nuclei. 6-16 mRNA synthesis can be detected as early as 5 minutes following the interferon alpha treatment; its synthesis in the nucleus of the cell peaks at three hours and decreases to basal levels within 8-12 hours (Friedman, 1984, Kelly, 1986). However, the mRNA of 6-16 is post-transcriptionally regulated and its presence in the cytoplasm can be detected even 12 hours after the nuclear synthesis has ceased (Friedman, 1984). The tight regulation of the 6-16 promoter has been used as a tool to obtain IFN-resistant mutants by linking the 6-16 promoter to selectable marker genes that also allow for back selection in case of low level deregulated expression (Pellegrini, 1989).

We have cloned and characterized the promoter region of 6-16 (Porter, 1988), its cis-acting IFN-regulatory sequences and the trans-acting factors with which they interact by 5' deletion analysis, transfection experiments, DNase-footprinting, gel retardation, in vitro transcription, and linking the promoter to reporter genes (Dale, 1989, Porter, 1988), (Chernajovsky, 1989).

The 6-16 promoter contains a direct repeat of 40 nucleotides (from -168 to -89) which includes the IFN-regulatory sequence that can confer IFN inducibility to heterologous promoters (Chernajovsky, 1989, Porter, 1988). This region binds a nuclear protein(s) of a molecular weight of 55 kilodaltons and can form in vitro a DNA-protein complex which is IFN modulated (Chernajovsky, 1990).

Another cis-acting element located at -450 binds constitutively to a nuclear protein of 80 kilodaltons and acts to down regulate expression of this promoter in conjunction with the direct repeat. By itself, this element seems not able to exert any transcriptional function.



-31-

In addition, the promoter of 6-16 has a CCAAT box located in the non coding strand of the promoter and a TATA box (Porter, 1988) (Chernajovsky, 1989) (Chernajovsky, 1990).

5

## 2. Selection of Lethal Genes

Lethal genes that may be used in accordance with the invention include genes encoding a variety of proteins that inhibit or arrest intracellular metabolism, and in particular, those leading to inhibition of key cellular functions such as DNA synthesis, transcription and translation, or those that paralyze other necessary cellular functions. Exemplary lethal genes that may prove useful in accordance with the invention include for example, the genes encoding ricin beta chain, pertussis toxin, etc. As discussed below, selection of a particular lethal gene will often be governed by the physiologic characteristics of the cell to which it will be introduced.

For some rapidly growing tumors or cells, an agent that will specifically inhibit DNA synthesis would offer an advantage. However, for others, e.g., solid tumors that grow slowly and do not proliferate very actively, such a DNA synthesis inhibitor may be less useful. In such cases, an agent that can specifically block another metabolic function of the cell, such as protein translation, could prove more relevant. Examples of lethal gene systems designed to address each of those cell types are discussed below.

For DNA synthesis inhibition, a preferred gene encodes the enzyme thymidine kinase from Herpes simplex virus. That enzyme is able to actively phosphorylate the thymidine analog gancyclovir (GANC) (1-(-deoxy-2-fluoro- $\beta$ -D-arabinofuranosyl)-5-ethyluracil), which after conversion to the 5' triphosphate by cellular kinases, inhibits DNA polymerase (Mansour, 1988, Mansuri, 1987).

The basis for the discriminating effect of GANC on cells expressing HSV-tk and the cellular tk enzyme, is the fact that the substrate requirements of the HSV-tk protein are less stringent than those of the cellular tk. Thus, cells  
5 expressing HSV-tk are sensitive to the toxic effects of GANC; normal cells are spared.

For protein synthesis inhibition, a preferred gene is that encoding the  $\alpha$  chain of Diphtheria toxin (DT-A). DT-A ADP-ribosylates elongation factor 2, producing protein  
10 synthesis inhibition and cell death. This gene has been used successfully in transgenic mice to specifically ablate pancreatic acinar cells when linked to the enhancer/promoter of the elastase I gene (Palmiter, 1987).

15           3.    Construction of Selectively Inducible Lethal Gene Constructs

The following prophetic examples describe construction of genetic constructs suitable for use in transfection of selected cells in accordance with the  
20 invention.

a.    Construction of a Herpes Simplex virus tk gene driven by the 6-16 promoter

A Bgl II-Pvu II (1775 bp) fragment from the plasmid  
25 pAGO containing all of the coding and polyadenylation signals of the tk gene (with 57 nucleotides of 5' untranslated region) is isolated and cloned together with the XhoI -HpaII (2.3Kb) fragment of the 6-16 promoter, which spans all of the promoter region up to nucleotide -  
30 4. The cloning is done in three steps. First, the BglIII to PvuII fragment of the HSV tk is subcloned into the BamHI-SmaI site of pUC18 to produce a plasmid named TK.pr. A diagnostic restriction carried with SmaI and EcoRI should yield two fragments, one of 360 and another one of  
35 4 Kb.

Then, the fragment XhoI (preceded by the polylinker sites HindIII to SalI of pUC18) to HpaII of the 6-16

-33-

promoter is subcloned into the AccI to HindIII sites of pUC18 to produce plasmid 6-16-XhoI-HpaII. Finally, the promoter of 6-16 is cloned in front of the tk gene by isolation of the Hind III-XbaI (2.3 kb) fragment from the  
5 plasmid 6-16.XhoI-HpaII into the XbaI-HindIII site of TK.pr to yield plasmid 6-16 TK, which contains the tk gene under the 6-16 promoter control.

10                   b.    Construction of a diphtheria toxin  $\alpha$  gene driven by the 6-16 promoter

A similar approach to the cloning of the 6-16.TK is used to produce a DT-A gene driven by the 6-16 promoter. First, the 795 bp Bgl II fragment (flanked by EcoRI and Hind III sites) is subcloned from the plasmid pDT-A  
15 (kindly provided by Dr. I Maxwell, University of Colorado). pDT-A contains the full coding sequence of the diphtheria  $\alpha$  toxin gene. Because the toxin polypeptide is a polyprotein having both the  $\alpha$  (24 kilodaltons) and  $\beta$  (38 kilodaltons) chains (Maxwell, 1987, Leong, 1983), the DNA  
20 is modified as follows to express the  $\alpha$  chain only.

The initiator methionine codon is substituted from GTG to ATG, and the first two amino acids are changed to Asp-Pro. In addition, the DNA fragment contains at the carboxy terminal end a synthetic Ser-Leu -stop codon from  
25 small SV40 t antigen with a splice signal but devoid of polyadenylation signal (Maxwell, 1986, Palmiter, 1987).

To clone the DT-A insert, the DT-A is digested with HindIII, and then ligated to a 940 bp HindIII-EcoRI SV40 fragment containing the polyadenylation sequences of T  
30 antigen, the remainder of the insert is recut with EcoRI. Consequently, the insert contains EcoRI sites at the ends, and the DT-A fragment has a polyadenylation signal 3' of it. This EcoRI 1.7 kb fragment is cloned into the EcoRI site of the 6-16 promoter plasmid.

35                   The orientation of the insert is assessed by restriction with BglII. There are three BglII sites, one

at -603 in the 6-16 promoter and two at each end of the DT-A 795 bp fragment. When the insert is in the right orientation, BglII digestion should release the 600 fragment of the 6-16 promoter till the BglII of the DT-A insert and the 795 by DT-A insert and the rest of the plasmid. In the opposite orientation, the fragments released will be of 795 (DT-A fragment) and 1.5 kb (containing the 940 bp SV40 3' and polyadenylation sequences + the 603 bp of the 6-16 promoter and the rest of the plasmid.

#### 4. Introduction of the Selected Genes Into the Target Cell

The selected immunopotentiating and lethal genes may be introduced into the cell by any of the methods known to those of skill in the art. However, in a preferred embodiment, cells will be transfected by the calcium phosphate coprecipitation method (Graham, 1973), lipofection, or electroporation. Tumor cells containing selected transfected immunopotentiating genes, e.g. HA or cytokines, may then be transfected with the lethal genes, or, conversely, cells can first be transfected with the lethal gene construct. Preferably, in addition to the lethal gene, the lethal gene construct will contain suitable marker and selection genes, which allow detection and selection of transfectants. Preferred among these are the marker plasmid pHCl10 (Hall, 1983), containing the bacterial  $\beta$ -galactosidase gene, and the selectable marker pSV2hygro (Blochlinger, 1984). Cells are transfected with those markers at a molar ratio of 15:15:1 and selected in 400 $\mu$ g/ml hygromycin B. In addition, selected clones are sensitive to gancyclovir or IFN and stain positively with X-gal (Price, 1987, Thompson, 1989).

### 5. Preclinical Studies

Because the molecular mechanisms of toxicity of the diphtheria toxin and gancyclovir are different, a number of different assays may be performed in order to determine the concentrations of gancyclovir or IFN needed to obtain the cytotoxic response in different cell types. The biological assays described below will be performed.

10                   a.   Assessment of concentration of Gancyclovir necessary for killing cells transfected with the Wild Type tk

                  The concentration of gancyclovir known to affect (by more than 90%) the plating efficiency of mouse embryonal-stem cells containing one copy of the HSV tk gene, is in the range of  $10^{-6}$  to  $5 \times 10^{-6}$  M. The concentration of gancyclovir needed to kill more than 90% of HSV-tk transfected tumor cells will be determined by plating efficiency assays and assays of [ $^3$ H]-thymidine incorporation. The plating efficiency assay is conducted by plating  $10^4$  cells in 10 cm<sup>2</sup> plates, with increasing concentrations of gancyclovir. Every three days the media is changed, and fresh gancyclovir added. After 9 days, the cells are stained with Giemsa, and the number of colonies per plate is determined. The assay of [ $^3$ H]-thymidine incorporation is performed similarly to the assay described below for biological fluids but using the HSV-tk tumor transfected cells.

30                   b.   Assessment of concentration of Gancyclovir and IFN necessary for killing cells transfected with the 6-16 tk ablation vector

                  The incorporation of GANC into DNA will probably depend on both its extracellular concentration and the intracellular concentration of the HSV tk enzyme. Cells having increased production of tk induced by addition of IFN may have enhanced sensitivity to GANC. This synergy is studied using increasing concentrations of both

cytotoxic agents in conditions in which the concentration of one of them remains constant. Increased toxicity will be assessed both by [<sup>3</sup>H]-thymidine incorporation and plating efficiency assays.

5

c. Determination of gancyclovir concentrations in biological fluids

In order to be able to rapidly assess the concentration of gancyclovir in biological samples such as serum, [<sup>3</sup>H]-thymidine incorporation assays are set up with LTK cells transfected with the HSV tk gene and selected in HAT media. The assay is carried out in the presence of aminopterin (1.8 ng/ml) and hypoxanthine (1.36 ng/ml) in order to avoid the synthesis of dTMP from dUMP by thymidine synthetase but to allow the synthesis of AMP and GMP through the HGPRT purine salvage pathway. Under these conditions the incorporation of [<sup>3</sup>H]-thymidine will depend mainly on the amount of inhibition of DNA synthesis produced by gancyclovir after its phosphorylation and processing to a triphosphate derivative that inhibits DNA polymerase, and the incorporation of [<sup>3</sup>H]-thymidine will be independent of the internal UMP/TMP pools. The fact that these cells do not contain an endogenous mammalian tk gene will be of great advantage because the cells will be extremely sensitive to gancyclovir.

The assay for DNA synthesis is performed as follows. Cells are seeded at  $15-25 \times 10^3$  cells per well in a 96-well microplate. The cells are incubated in triplicate with or without known concentrations of gancyclovir in the presence of hypoxanthine and aminopterin. After 24-28 hrs, cells are labeled with [<sup>3</sup>H]-thymidine 915 mCi/ml; 25 Ci/mmol; 1 Ci + 37 GBq; Amersham) for 1 hr. The cells are then washed twice with phosphate-buffered saline, treated with 5% trichloroacetic acid for 30 min at 4°C, and washed three times with 5% trichloroacetic acid. The precipitate is dissolved in 0.1 ml of 0.2 M NaOH at 37°C for 30 min

and neutralized by 0.01 ml of 2 M CH<sub>1</sub>; radioactivity is determined in a  $\beta$ -scintillation counter. The concentration of gancyclovir from animal fluids could be found by two fold dilutions of the samples and comparison of the extent of inhibition obtained to the inhibition curve of the standard used.

10                   d.    Assessment of concentration of IFN  
                      necessary for inhibition of protein  
                      synthesis and killing of cells transfected  
                      with the 6-16 DT-A plasmid

15                   To investigate the dose of IFN and time of exposure  
                      necessary to produce a cytotoxicity of more than 90%,  
                      inhibition of protein synthesis in cells transfected with  
                      the plasmid 6-16 DT-A is measured using a sensitive  
                      colorimetric assay.

20                   Tumor cells cotransfected with the 6-16 DT-A plasmid,  
                      pSV2neo (for selection in G418) and with pCH110, which  
                      contains the bacterial  $\beta$ -galactosidase enzyme under the  
                      control of the early SV40 promoter, are selected by  
                      culture in G418. Resistant cell clones are isolated and  
                      tested by southern blotting to prove that all plasmids are  
                      present. Selected cell clones are those which are killed  
                      by IFN and have detectable levels of  $\beta$ -galactosidase  
25                   activity.

                      The inhibition of protein synthesis due to the  
                      activation of the diphtheria toxin will produce a decrease  
                      in the amount of  $\beta$ galactosidase that can be detected in a  
                      colorimetric assay using the substrate pNPG (p-  
30                   nitrophenyl- $\beta$ -D-galactopyranoside). This substrate of  $\beta$ -  
                      galactosidase turns yellow upon cleavage by the enzyme and  
                      the accumulation of the yellow product can be measured at  
                      a wavelength of 220 nm in Elisa plates. This enzymatic  
                      system will also be useful in vivo. The presence of cells  
35                   containing the  $\beta$ -galactosidase gene can be visualized in  
                      histological samples by using the substrate X-gal that,  
                      upon cleavage, produces a blue precipitate in the cells.

Therefore, this assay allows the determination of the presence of tumor cells containing lethal gene constructs in situ in animal tissue after treatment with IFN.

5           6.   In vivo experiments

The following prophetic examples describe practice of the invention in a variety of murine tumor models.

CT-26 cells with or without immunopotentiating and/or lethal genes are injected into syngeneic BALB/c animals. Similarly, SP1 and RENCA cells described below are injected into their syngeneic hosts CBA and BALB/c mice, respectively. To estimate the immunogenic potential of the cells expressing the lethal genes,  $1 \times 10^3$  to  $10^7$  cells will be injected in the right flank of the animals while the parental nontransfected cells will be injected on the left flank. The number of tumor cells required to kill in 50% of the animals will be estimated by the method of Reed and Muench (Reed, 1938) as previously described (Fearon, 1988).

20

7.   Models

a.   H4A

The H4A cell line was derived by transfection of the SP1 mammary adenocarcinoma with the influenza virus hemagglutinin (HA) gene. Cells expressing HA were selected four times using Fluorescence Activated Cell Sorting (FACS) as previously described (Fearon, et al., 1989), and the H4A cell line was obtained. H4A fails to grow in syngeneic hosts at a challenge dose of  $1 \times 10^4$ , but does grow if  $1 \times 10^5$  H4A cells are injected subcutaneously. Despite the growth of the cells, the animals do mount a cytotoxic T cell response, though this response is inadequate or occurs too late to offset tumor growth.

35

CBA mice will be injected with H4A, or H4A transfected with either HSV-tk or DTA. When the resultant



-39-

tumor reaches different sizes (2-4 mm, 6-8 mm, 1 mm or 2 mm) all animals will be treated in the following manner to kill the injected cells.

	<u>TUMOR CELL INJECTED</u>	<u>TREATMENT</u>
5	H4A	None
	H4A	GANC
	H4A	IFN- $\alpha$
	H4A	GANC + IFN- $\alpha$
10	H4A - HSV tk	None
	H4A - HSV tk	GANC
	H4A - HSV tk	IFN- $\alpha$
	H4A - HSV tk	GANC + IFN- $\alpha$
	H4A-DTA	None
15	H4A-DTA	GANC
	H4A-DTA	IFN-a
	H4A-DTA	GANC + IFN-a

The initial doses of IFN-a (Hoffman-LaRoche, Nutley N.J.) will be  $5 \times 10^4$  units/injection s.c. three times a week for two consecutive weeks. GANC (Syntex, Palo Alto, CA) will be given s.c. at 20 mg/kg/day initially.

Animals in whom the primary tumor has been rejected are then:

- a) be assessed for a splenic cytotoxic T cell response;
- b) be challenged with  $1 \times 10^4$ ,  $1 \times 10^5$ , or  $5 \times 10^5$  parent cells to detect immunity to a parent tumor challenge.

#### 30 b. CT-26/IFN- $\gamma$

The CT-26 murine colon carcinoma, when transfected with and expressing IFN- $\gamma$ , becomes partially immunogenic. While a  $1 \times 10^5$  challenge dose of parental CT-26 cells is lethal to normal animals in 3 weeks; 60% of the CT-26 IFN- $\gamma$  cell injected animals survive greater than 8 weeks. These cells appear to be immunogenic, but the response

they engender is sometimes insufficient to prevent tumor growth. In addition, animals immunized with viable CT-26/IFN- $\gamma$  were not protected against a parent CT-26 challenge. Furthermore, irradiated CT-26/IFN- $\gamma$  cells were also nonprotective against a challenge with parental cells.

c. RENCA Model

RENCA is a renal cell carcinoma of BALB/c mice. The injection of  $5 \times 10^4$  RENCA cells into the subrenal capsule of syngeneic mice results in the development of a u 1 cm tumor with two weeks. If the tumor bearing kidney is surgically removed, the animals will all develop extensive lung metastases within two weeks.

C. Human Immunotherapy

Due to the necessary constraints imposed on human therapy, the present invention has not yet been tested in human subjects. However, it is contemplated that the invention may be used in humans having selected tumors, for example, of the colon, breast, ovary, melanoma, and sarcoma. Generally, it is preferred to select for treatment patients who have had a discrete primary tumor removed for which there is a high risk of recurrence. In a usual situation, tumor cells from the patient will be isolated and transfected with suitable immunopotentiating and lethal genes. Of course, it will be appreciated that when the immunopotentiating gene is a cytokine, such as interleukin-2, a gene encoding a human cytokine will be used. After transfection, the cells will be selected to obtain cell lines which are stably transfected with the selected genes.

In the ideal situation, the patient will first be treated with any of a number of regimes designed to reduce tumor burden, such as surgical resection, irradiation, etc., prior to administration of the tumor cell immunogen.

-41-

The cells are then allowed to immunize the host for a selected period of time, usually about 14 days. Throughout this period of time, suitable assays may be performed to assess the immune status of the patient to the particular tumor in question. However, it should be realized that where the immunization is local in nature, systemic immunity may not be observed in all cases, and it may thus be necessary to obtain lymphocytes from the tumor site for such studies. In any event, after a selected period of time in which the host has been effectively immunized against its tumor cells, the lethal gene will be caused to eliminate the remaining immunizing tumor cells. In the instance when the selectably inducible promoter employed is a 6-16 promoter described here, alpha interferon will be administered to the patient in a dose effective to initiate transcription of the lethal gene but not so high as to be acutely toxic to the patient. The alpha interferon may also be administered locally to the site of the vaccine introduction. When the Herpes simplex thymidine kinase gene is used, it will be necessary to administer gancyclovir. When the thymidine kinase gene is driven by the 6-16 promoter, administration of both gancyclovir and interferon alpha will be warranted. While definite parameters for each of these administrations and the like have not yet been ascertained, they should be readily ascertainable by those of ordinary skill in the art with the assistance provided here.

30

\* \* \* \* \*

35

The foregoing description of the invention has been directed to particular preferred embodiments in accordance with the requirements of the patent statutes and for purposes of explanation and illustration. It will be apparent, however, to those skilled in the art that many

-42-

modifications and changes may be made without departing from the scope and the spirit of the invention.

For example, numerous methods for introducing the exogenous genes into the target cells may be used. In addition, various selected genes may be transfected in accordance with the invention. For example, certain aspects of the invention, such as the provision of a selectively inducible lethal gene, may not be limited to use in immunotherapy but may be more widely applicable to any situation in which it is desirable to be able to specifically ablate a genetically engineered cell in vivo. In addition, the transfected cell lines of the present invention are useful for ex vivo immunization, for example, in activation of LAK cells for infusion into a patient according to the procedure described in U.S. Patent # 4,690,915, issued to Rosenberg, or in vitro activation of cytotoxic T lymphocytes or for promoting growth of cytokine dependent cell lines, such as IL-2 dependent cell lines. The ability to purge in vitro cultures of the transfected cells by simply inducing transcription and translation of the lethal gene provides an additional advantage. Therefore, it is apparent that the invention may also be utilized, with any number of suitable modifications within the state of the art. It is the Applicants intention in the following claims to cover all such equivalent modifications and variations which fall within the true spirit, and scope of the invention.

#### REFERENCES

The following references may facilitate understanding or practice of certain aspects of the present invention. Inclusion of a reference in this list is not intended to and does not constitute an admission that such reference represents prior art with respect to the present invention.

- Battacharya et al., J. Immunol. 127:2488-2495.
- Blochlinger and Diggelman, Mol. Cell. Biol. 4:2929-2931 (1984).
- 5 Boon, Immunology Today 6:307-311 (1985).
- Brattain, et al., Cancer Res. 40:2142-2146 (1980).
- 10 Brenner, et al., Adv. Immunol. 43:133-192 (1988).
- Cantor and Boyse, J. Exp. Med. 141:1390-1399 (1975).
- Chernajovsky et al., Lymphokine Res. 9:199-212 (1990).
- 15 Chernajovsky, FEBS. Lett. 258(2):323-30 (1989).
- Colbere-Garapin et al., Proc. Natl. Acad. Sci. USA 76:3755-3759 (1979).
- 20 Dale et al., Proc. Natl. Acad. Sci. USA 86(4):1203-7 (1989).
- Dialynas et al., J. Immunol. 131:2445-2452 (1983).
- 25 Fearon et al., Cancer Res. 48:2975-2980 (1988).
- Fearon et al., Cell 60:397-403 (1990).
- 30 Felgner et al., Proc. Natl. Acad. Sci. 84:7413 (1987).
- Fidler, Cancer Res. 35:218-234 (1975).
- Friedman et al., Cell 38:745-755 (1984).
- 35 Frost and Chernajovsky, Transformation injury and the unicellular phenotype of malignant cells 9:93-98 (1990).
- 40 Frost et al., Cancer Res. 47:2690 (1987).
- Graham and van der Eb, Virology 52:456-467 (1973).
- Grimm et al., J. Exp. Med. 155:1823-1831 (1982).
- 45 Hall et al., J. Molecular and Applied Genetics 2:101-109 (1983).
- Herberman, NK Cells and Other Natural Effector Cells, New York, Academic Press (1982).
- 50 Hewitt et al., Br. J. Cancer 33:241-259 (1976).
- Hui et al., Nature 311:750-752 (1984).

- Itaya et al., Cancer Immunol. Immunother. 28:248-252 (1989).
- Janis et al., Science 244:713-715 (1989).
- 5 Jenkins et al., Proc. Natl. Acad. Sci. USA 84:5409-5413 (1987a).
- Jenkins et al., Immunol. Rev. 95:113-135 (1987b).
- 10 Julius et al., Eur. J. Immunol. 3:645 (1973).
- Karasuyama and Melchers, Eur. J. Immunol. 18:97-104 (1988).
- 15 Karasuyama et al., J. Exp. Med. 169:13-25 (1989).
- Keene and Forman, J. Exp. Med. 155:768-782 (1982).
- 20 Kelly et al., Embo. J. 5(7):1601-6 (1986).
- Kerbel, Amer. J. Path. 97:609 (1979).
- Kerbel et al., Proc. Natl. Acad. Sci. 84:1263 (1987).
- 25 Kiessling et al., Int. J. Cancer 15:933-940 (1975).
- Leong et al., Science 220:515-517 (1983).
- 30 Lo et al., Cell 53:159-168 (1988).
- Lurquin et al., Cell 58:293-303 (1989).
- Mansour et al., Nature 336:348-352 (1988).
- 35 Mansuri et al., J. Med. Chem. 30:867-871 (1987).
- Markman et al., Nature 336:476-479 (1988).
- 40 Maryanski et al., Nature 324:578-579 (1986).
- Maxwell et al., Mol. Cell. Biol. 7:1576-1579 (1987).
- Maxwell et al., Cancer Res. 46:4660-4664 (1986).
- 45 Mitchell, Proceedings of the Second Conference on Immunity to Cancer, Williamsburg, VA, Nov. 9-11, 1987; 288:323-336 (1989).
- 50 Möller, Immunol Rev. 51 (1980).
- Moore et al., Cell 54:777-785 (1988).
- Mory et al., Eur. J. Biochem. 120:197 (1981).

- Mulligan and Berg, Science 209:1422-1427 (1980).
- Murphy and Haushesky, J. Natl. Cancer Inst. 50:1013-1025 (1973).
- 5 Ortaldo et al., J. Exp. Med. 164:1193-1205 (1986).
- Ozato et al., J. Immunol. 125:2473-2477 (1980).
- 10 Ozato et al., Transplantation 34:113-120 (1982).
- Palmiter et al., Cell 50:435-443 (1987).
- Pardoll et la., FASEB J. 1:103-109 (1987).
- 15 Pellegrini et al., Mol. Cell Biol. 9(11):4605-12 (1989).
- Phillips and Lanier, J. Exp. Med. 164:814-825 (1986).
- 20 Porter et al., Embo J. 7(1):85-92 (1988).
- Price et al., Proc. Natl. Acad. Sci. USA 84:156-160 (1987).
- Raulet, Annu. Rev. Immunol. 7:175-208 (1989).
- 25 Reed and Muench, Am. J. Hyg. 27:493 (1938).
- Sarmiento et al., J. Immunol. 125:2665-2672 (1980).
- 30 Sella et al., Clin. Exp. Metastasis 7:97-105 (1989).
- Sitkovsky and Paul, Nature 322:306 (1988).
- Smith et al., J. Bacteriol. 141:184-189 (1980).
- 35 Tanaka et al., Science 228:26-30 (1984).
- Tepper et al., Cell 57:503-512 (1989).
- 40 Thompson et al., Cell 56:917-310 (1989).
- Townsend et al., Cell 44:959-968 (1986).
- Trenn et al., J. Immunol. 140:1101-1106 (1988).
- 45 Van Pel and Boon, Proc. Natl. Acad. Sci. USA 79:4718-4722 (1982).
- vonBoaehmer and Haas, J. Exp. Med. 150:1134-1142 (1979).
- 50 Wallich et al., Nature 315-301-305 (1985).
- Widmer et al., J. Exp. Med. 166:1447-1455 (1987).

Wigler et al., Proc. Natl. Acad. Sci. USA 76:1373-1376  
(1979).

Wiltrout et al., J. Natl. Cancer Instit. 61:183 (1978).

5

Zinkernagel et al., J. Exp. Med. 147:897-911 (1978).



## CLAIMS:

1. A composition for potentiating the immune response to a tumor, the composition comprising a cell derived from the tumor, the cell including a first exogenous gene  
5 encoding an immunopotentiating polypeptide and a second exogenous gene including a promoter in operative linkage to DNA encoding a polypeptide capable of killing the cell upon induction of the promoter.
- 10 2. The composition of claim 1 wherein the promoter is a selectively inducible promoter.
3. The composition of claim 2 wherein the inducible promoter is a 6-16 interferon inducible promoter.
- 15 4. The composition of claim 2 or claim 3 wherein the second exogenous gene includes a selectively inducible promoter in operative linkage to a gene encoding the alpha chain of diphtheria toxin.
- 20 5. The composition of claim 2 or claim 3 wherein the second exogenous gene includes a selectively inducible promoter in operative linkage to a gene encoding Herpes simplex virus thymidine kinase.
- 25 6. The composition of claim 1, claim 2, or claim 3, wherein the immunopotentiating polypeptide is a cytokine.
7. The composition of claim 6 wherein the  
30 immunopotentiating polypeptide is an interleukin.
8. The composition of claim 7 wherein the interleukin is interleukin-1.
- 35 9. The composition of claim 7 wherein the interleukin is interleukin-2.

10. The composition of claim 7 wherein the interleukin is interleukin-3.
- 5 11. The composition of claim 7 wherein the interleukin is interleukin-4.
12. The composition of claim 7 wherein the interleukin is interleukin-5.
- 10 13. The composition of claim 7 wherein the interleukin is interleukin-6.
14. The composition of claim 1, 2, or 3 wherein the immunopotentiating polypeptide is an antigen alien to the  
15 host of the tumor.
15. The composition of claim 14 wherein the antigen is a histocompatibility antigen.
- 20 16. The composition of claim 14 wherein the antigen is a viral envelope protein antigen.
17. The composition of claim 14 wherein the antigen is a bacterial antigen.  
25
18. The composition of claim 17 wherein the antigen is the 65kDa antigen of Mycobacterium tuberculosis.
19. The composition of claim 1 wherein the genome  
30 includes a third exogenous gene, encoding a second immunopotentiating polypeptide.
20. The composition of claim 1 wherein the second  
35 exogenous gene encodes an immunopotentiating antigen if the first exogenous gene encodes an immunopotentiating cytokine and wherein the second exogenous gene encodes an

immunopotentiating cytokine if the first exogenous gene encodes an immunopotentiating antigen.

5 21. A cell from a vertebrate organism comprising a first exogenous gene encoding a selected polypeptide and a second exogenous gene comprising a promoter in operative linkage to DNA encoding a second selected polypeptide, the second selected polypeptide capable of killing the cell.

10 22. The cell of claim 21 wherein the promoter is a selectively inducible promoter and the second polypeptide is capable of killing the cell upon induction of the promoter.

15 23. The cell of claim 22 wherein the promoter is 6-16 interferon promoter.

20 24. The cell of claim 21 wherein the second polypeptide is capable of killing the cell upon addition of a substrate for the polypeptide.

25 25. A method for producing a cell for gene therapy of a selected disease comprising:

- 25 a. introducing a first exogenous gene encoding a selected polypeptide into a cell; and
  - b. introducing a second exogenous gene containing a promoter in operative linkage with DNA encoding a polypeptide capable of killing the cell into the cell to produce a cell having both the first and the second exogenous genes maintained in the cell.
- 30

35 26. The method of claim 25 wherein said promoter is a selectively inducible promoter.

27. The method of claim 26 comprising the additional step of inducing the promoter so as to kill the cells.

5 28. A method for gene therapy of a vertebrate organism comprising administering the composition of any one of claims 21 -27 to the organism.

29. A method for gene therapy of a vertebrate organism comprising:

- 10           a. introducing a first exogenous gene encoding a selected polypeptide into a cell; and
- b. introducing a second exogenous gene containing a promotor in operative linkage with DNA encoding a polypeptide capable of killing the cell into
- 15           the cell to produce a cell having both the first and the second exogenous genes stably maintained in the cell; and
- c. administering the cell to the organism.

20 30. The method of claim 29 wherein the promoter is a selectively inducible promoter.

31. The method of claim 30 comprising the additional step of inducing the promoter.

25

32. A cellular preparation for potentiating an immune response of a vertebrate organism to a tumor comprising a preparation of cells, derived from the tumor, containing an exogenous gene encoding an immunopotentiating

30 interleukin.

33. A cellular preparation for potentiating the immune response of a vertebrate organism to a tumor comprising a preparation of cells, derived from the tumor, containing

35 an exogenous gene encoding interleukin-2.

-51-

34. The composition of claim 32 or claim 33, wherein the exogenous gene is introduced into the cells by transfection.

5 35. A composition for enhancing non-specific resistance of a vertebrate organism to growth of a tumor comprising a preparation of cells including an exogenous gene encoding interferon.

10 36. The composition of claim 35 wherein the interferon is interferon  $\gamma$ .

37. The composition of claim 35 wherein the exogenous gene is introduced into the tumor by transfection.

15 38. The composition of claim 32 or claim 35 wherein the cells further comprise an exogenous gene encoding an additional immunopotentiating polypeptide.

20 39. The composition of claim 38 wherein the additional immunopotentiating polypeptide is an antigen foreign to the mammal.

25 40. The composition of claim 38 wherein the antigen is a viral envelope protein.

41. The composition of claim 38 wherein the antigen is a bacterial cell antigen.

30 42. The composition of claim 38 wherein the antigen is the 65kDa antigen of *Mycobacterium tuberculosis*.

43. The composition of claim 38 wherein the immunopotentiating polypeptide is a cytokine.

35

44. The composition of claim 38 wherein the cytokine is interleukin-1.

5 45. The composition of claim 38 wherein the cytokine is interleukin-3.

46. The composition of claim 38 wherein the cytokine is interleukin-4.

10 47. The composition of claim 38 wherein the cytokine is interleukin-5.

15 48. The composition of claim 38 wherein the cytokine is interleukin-6.

49. The composition of claim 38 wherein the cytokine is interferon.

20 50. The composition of claim 38 wherein the cytokine is gamma interferon.

25 51. A method for potentiating the immune response of a vertebrate organism to a tumor comprising the steps of introducing into the organism a viable preparation of the composition of any one of claims 32-37.

30 52. A method for potentiating the immune response of a vertebrate organism to a tumor comprising the steps of introducing into the organism a viable preparation of the composition of claim 38.

35 53. The method of claim 51 comprising the additional steps of reducing the number of tumor cells in the vertebrate organism prior to introducing the preparation.

54. The method of claim 53 wherein the reduction comprises surgical resection.

Table 1. Growth of CT26, CT26-IL-2<sup>+</sup>, and CT26-neo-IL-2<sup>-</sup> in BALB/c Mice

5			# Mice with Tumor/ # Mice Injected
	Tumor	Injection Dose <sup>a</sup>	
10	CT26	1 x 10 <sup>3</sup> cells	9/10 <sup>b</sup>
		1 x 10 <sup>4</sup> cells	20/20 <sup>c</sup>
		1 x 10 <sup>5</sup> cells	25/25 <sup>c</sup>
		1 x 10 <sup>6</sup> cells	20/20 <sup>c</sup>
15	CT26-IL-2 <sup>+</sup>	1 x 10 <sup>4</sup> cells	0/20 <sup>c</sup>
		1 x 10 <sup>5</sup> cells	0/35 <sup>c</sup>
		1 x 10 <sup>6</sup> cells	2/55 <sup>c</sup>
		1 x 10 <sup>7</sup> cells	4/15 <sup>c</sup>
20	CT26-neo-IL-2 <sup>-</sup>	1 x 10 <sup>4</sup> cells	10/10 <sup>b</sup>
		1 x 10 <sup>5</sup> cells	20/20 <sup>d</sup>
		1 x 10 <sup>6</sup> cells	10/10 <sup>d</sup>

<sup>a</sup> BALB/c mice were injected subcutaneously on the left hind leg, and tumor growth was observed.

25 <sup>b</sup> All mice had tumors by 3 weeks.

<sup>c</sup> All mice had tumors by 2 weeks.

<sup>d</sup> Most mice had tumors by 2 weeks, all had tumors by 3 weeks.

<sup>e</sup> Tumor-free mice were observed from 6-12 weeks.

30



Table 2. In Vivo Growth, Protective Immunity, and CTLs Generated by IL-2-Transfected B16 Melanoma Cells

5	10	# Cells Injected <sup>b</sup>	Tumor Growth <sup>c</sup>	Protection	Lysis
				against Challenge with B16 <sup>d</sup>	of B16 <sup>e</sup>
15	B16	1 x 10 <sup>4</sup>	4/5	---	---
		1 x 10 <sup>5</sup>	5/5	---	16%
		1 x 10 <sup>6</sup>	5/5	---	---
		B16-IL-2 <sup>+</sup> 1 x 10 <sup>4</sup>	0/9	9/9	---
		1 x 10 <sup>5</sup>	0/8	6/8	26%
		1 x 10 <sup>6</sup>	0/9	5/9	---
20					

<sup>a</sup> B16-IL-2<sup>+</sup> transfectant was produced by transfection with pBCMG-neo-mIL-2 as described in Experimental Procedures.

25 <sup>b</sup> Cells were injected subcutaneously into the left flank as in Table 1.

<sup>c</sup> Tumor growth was measured weekly. Animals were observed for 7 weeks. All animals that developed tumors did so by 2 weeks after injection.

30 <sup>d</sup> Challenge with the 1 x 10<sup>5</sup> parental B16 cells was done in the right flank 2 weeks after immunization with B16-IL-2<sup>+</sup> cells as in Figure 3. Animals were observed for 7 weeks after challenge. Numbers represent animals that acquired tumors. These tumors were observed to arise between 3 and 5 weeks after challenge. Tumor-free animals were observed for 7 weeks.

35 <sup>e</sup> CTL assays were performed after a 5 day stimulation with mitomycin C-treated B16 cells as in Figure 1a. Numbers represent % specific lysis after an 8 hr <sup>51</sup>Cr release assay at 100:1 effector to target ratio.

40

TABLE 3

5	Growth of IFN $\gamma$ Transfected SP1 Tumor Cells				
	IFN $\gamma$ Production (Units/ml)	No. of mice with tumor/ No. of mice challenged			
10		$10^5$	$5 \times 10^5$	$10^6$	
15	SP1 Parent SP1/IFN $\gamma$	---	10/10	NT	NT
	1C	16	2/3*	NT	NT
20	3C	32	3/3*	NT	NT
	6L	256	0/15	0/10	1/15°
25	Groups of CBA mice were injected s.c. with either $10^5$ , $5 \times 10^5$ or $10^6$ cells from three different transfection plates (1C, 3C and 6L). The animals were then observed for tumor growth for 90 days.				
30	* Growth at 24 days				
	° Growth at 90 days				

-57-

TABLE 4

5 Induction of Cytotoxic T Cells by IFN $\gamma$  producing SP1 cells

10	Tumor cells used for immunization	Target Cells	Effector: Target Ratio	% Killing		
				Whole Spleen Cells	NWNA	NWA
15	CBA	CBA	100:1	2.3	---	---
	6L	CBA	100:1	30	---	---
20			50:1	19	19	64
			10:1	12	8	39
25	6L	6L	100:1	40	---	---
			50:1	16	24	60
			10:1	0	13	38
30	6L	MDW1	50:1	2.2	2.4	19
			10:1	1.8	2.7	8

35 Groups of CBA mice were injected s.c. with either  $5 \times 10^5$  SP1 or SP1/IFN $\gamma$  (6L) cells. Two weeks later the animals were killed and their spleen cells incubated with irradiated SP1 or 6L cells. Five days later the

40 lymphocytes were harvested and passed through a nylon wool column and adherent and nonadherent spleen cells were added to the <sup>111</sup>Inox labeled targets listed, at the ratios indicated.

NWA - Nylon Wool Adherent

45 NWNA - Nylon Wool Non-Adherent

TABLE 5  
Immunoprotection by IFN $\gamma$  transfected SP1 cells against  
a challenge with parent cells

5	Tumor	<u>Immunization dose</u>		<u>Challenge Dose</u>		
		1st	2nd	10 <sup>4</sup>	5x10 <sup>4</sup>	10 <sup>5</sup>
	SP1	---	---	10/10*	10/10	10/10
	SP1/IFN $\gamma$ 6L	2x10 <sup>6</sup>	10 <sup>6</sup>	9/18	10/10	18/19
10	CBA mice were immunized with 10 <sup>6</sup> 6L cells on day 0 and again on day 14. On day 19, immunized and control animals were challenged with 10 <sup>4</sup> or 10 <sup>5</sup> parent SP1 cells. The animals were then observed for tumor growth.					
15	* Number of mice with tumors/Number of mice challenged.					

TABLE 6  
Growth of IFN $\gamma$  producing CT-26 tumor cells

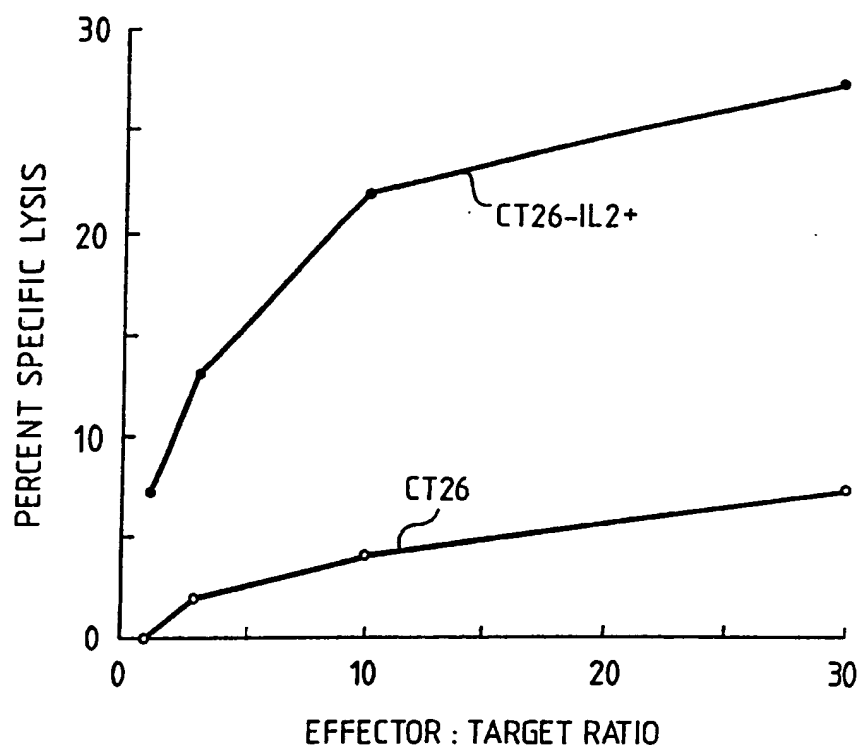
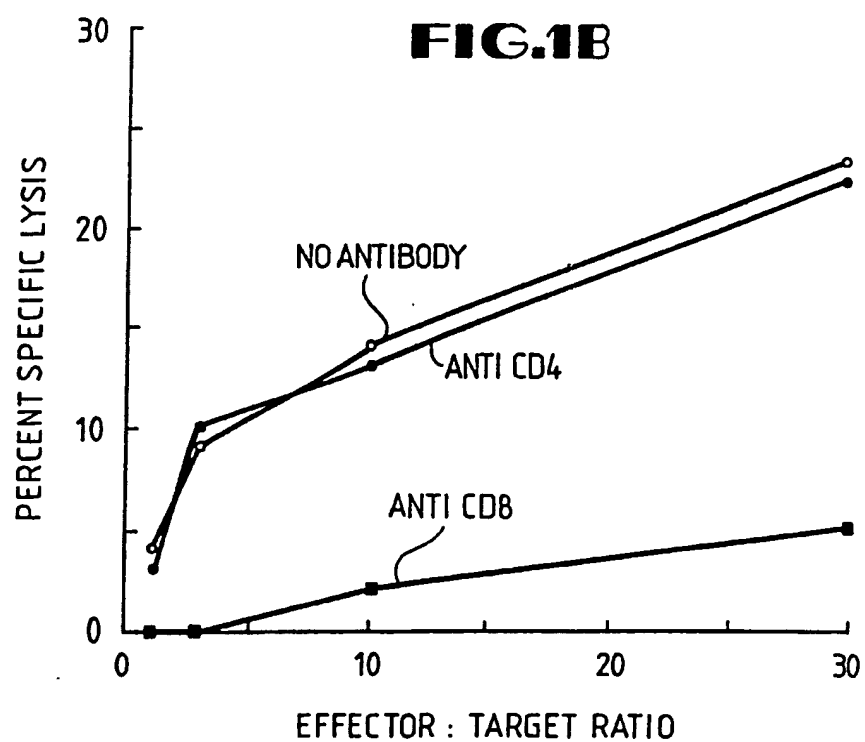
5	Tumor cells	Challenge dose	Route	Tumorigenicity		
				3w	5w	7w
10	CT-26	1x10 <sup>5</sup>	s.c.	9/10*	---	---
	CT-26/IFN $\gamma$	1x10 <sup>5</sup>	s.c.	2/15	---	9/15
	CT-26	5x10 <sup>4</sup>	i.v.	---	5/5	---
	CT-26/IFN $\gamma$	5x10 <sup>4</sup>	i.v.	---	0/5	3/5

Groups of BALB/c mice were injected s.c. or i.v. with CT-26 or CT-26IFN $\gamma$  cells and observed for tumor growth and survival.

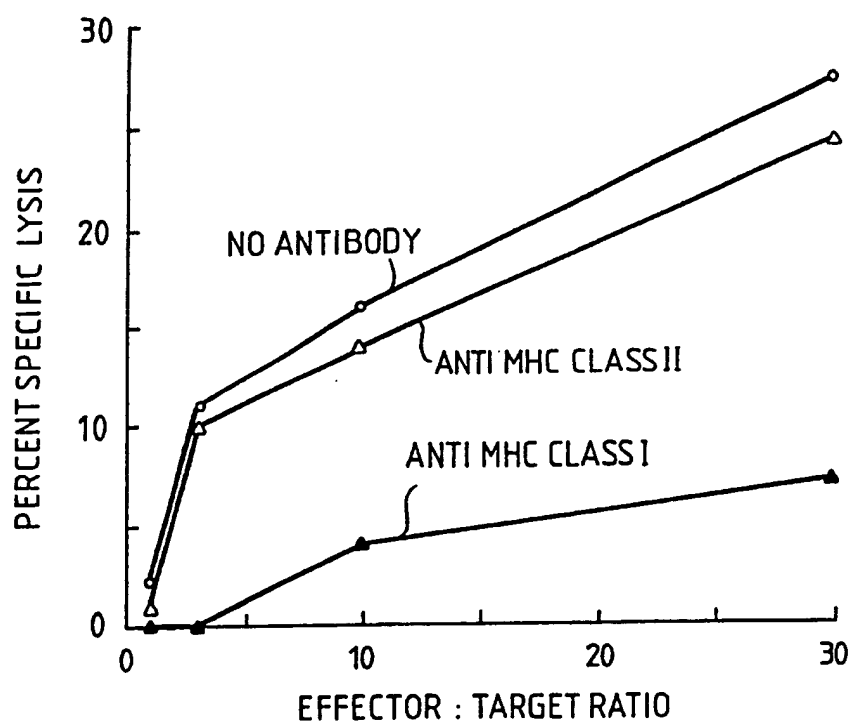
\* Number of mice with tumors/Number of mice challenged.

**FIG.1A**

1/7

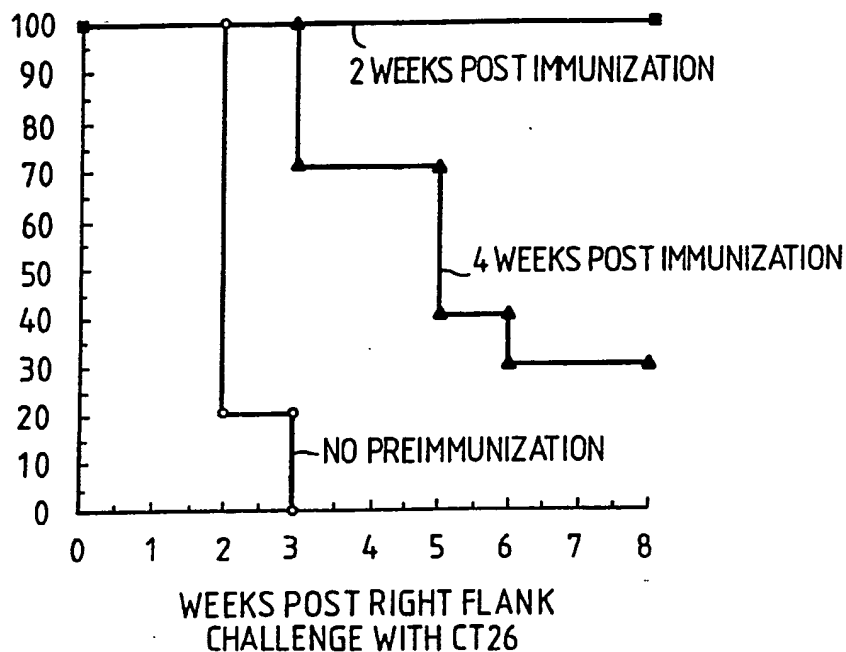
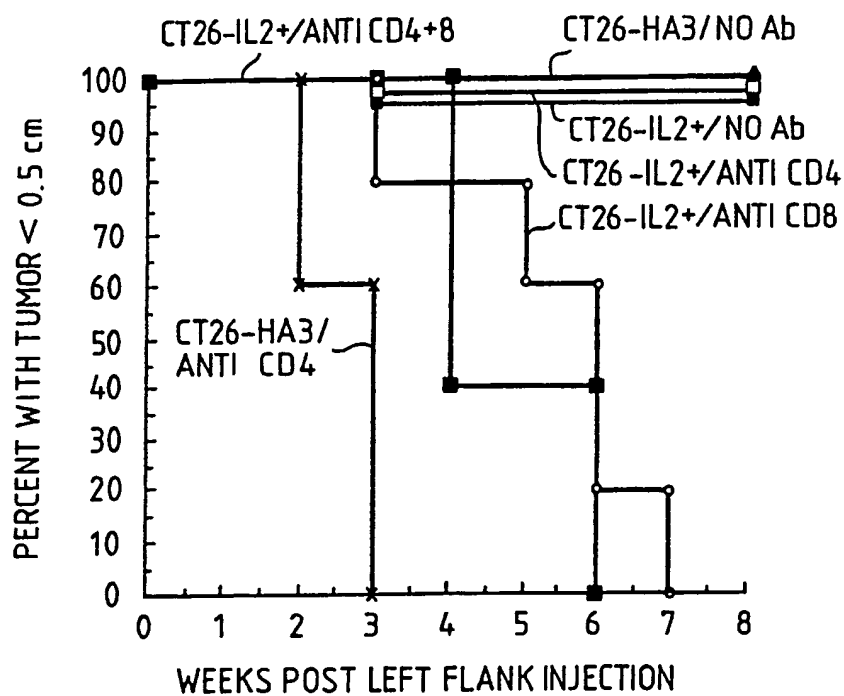
**FIG.1B**

2/7

**FIG.1C**

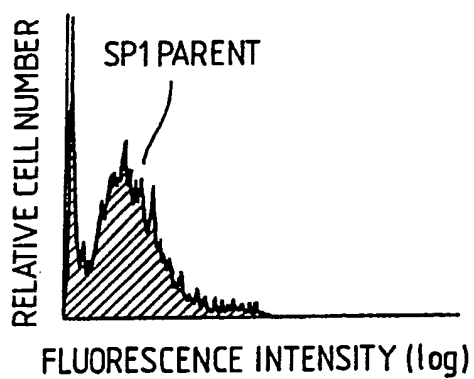
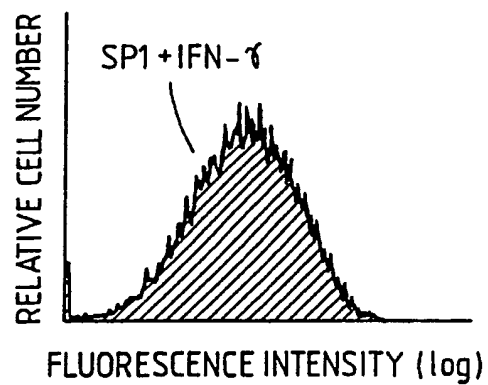
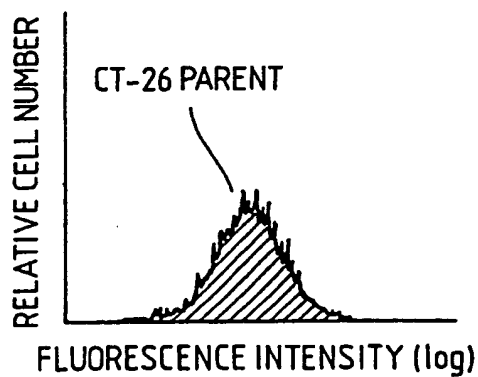
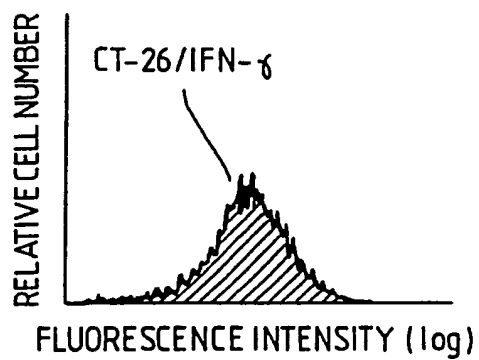
**FIG. 2**

3/7

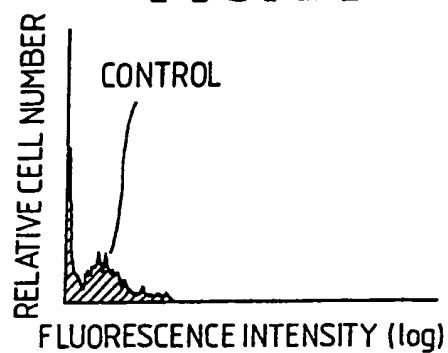
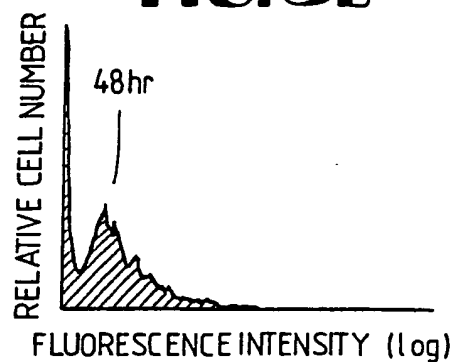
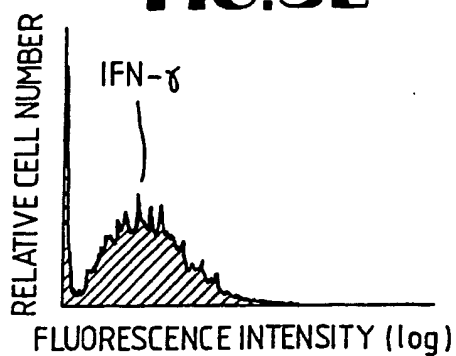
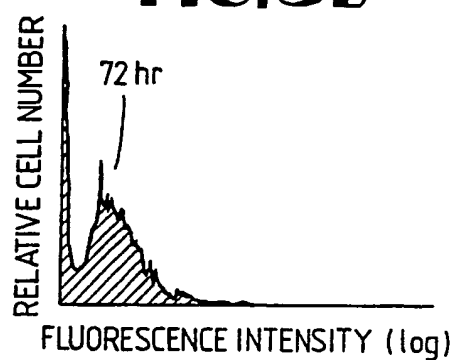
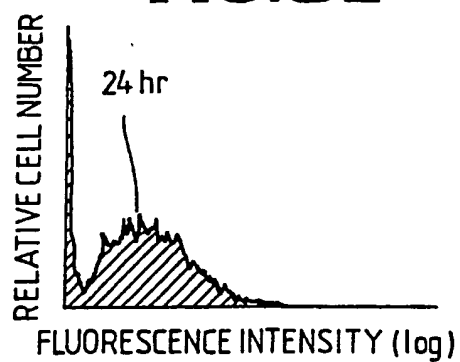
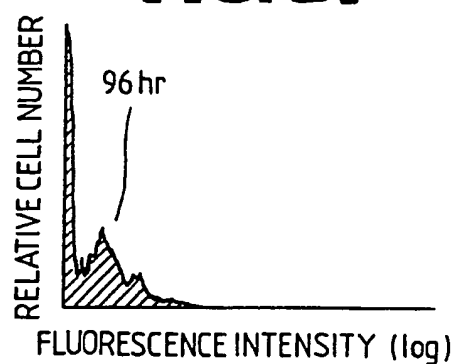
**FIG. 3**



4/7

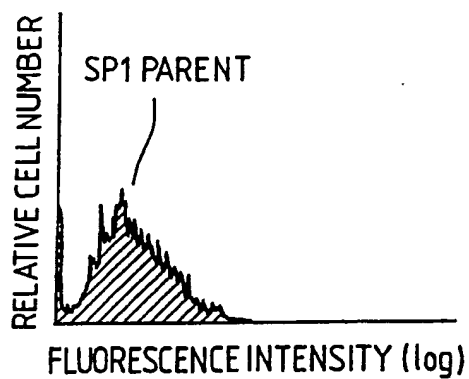
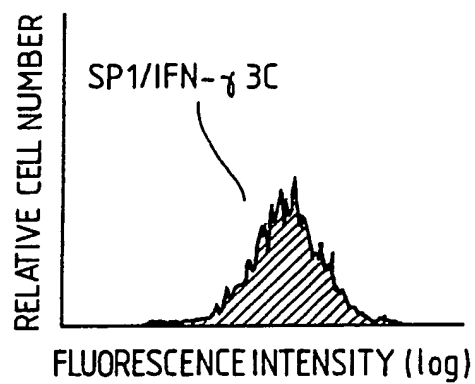
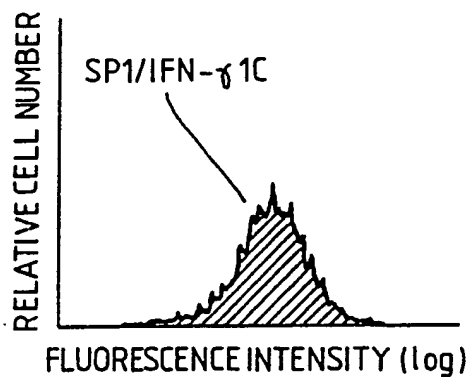
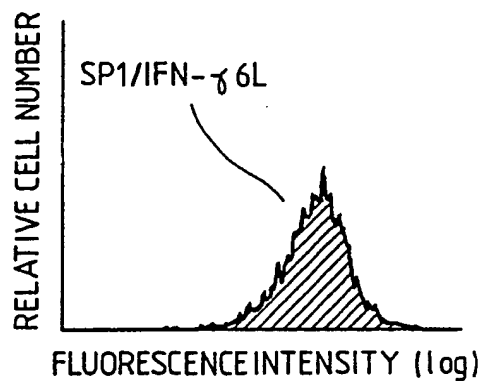
**FIG.4A****FIG.4B****FIG.4C****FIG.4D**

5/7

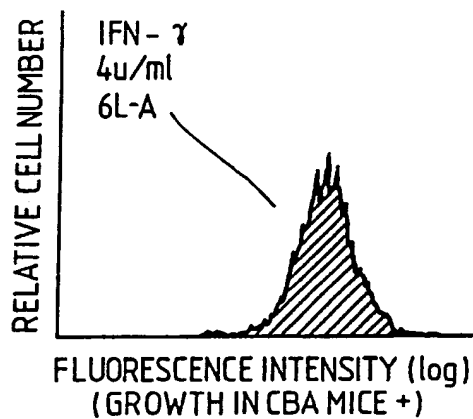
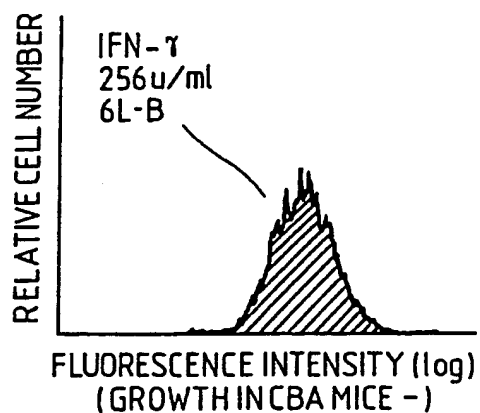
**FIG. 5A****FIG. 5B****FIG. 5C****FIG. 5D****FIG. 5E****FIG. 5F**

SUBSTITUTE SHEET

6/7

**FIG. 6A****FIG. 6B****FIG. 6C****FIG. 6D**

7/7

**FIG.7A****FIG.7B**

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/06612

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl.5 C 12 N 5/10 // C 12 N 15/19	C 12 N 15/85 A 61 K 48/00 C 12 N 15/31	A 61 K 35/12 C 12 N 15/54
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl.5	A 61 K                  C 07 K                  C 12 N	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>o</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Cell, volume 60, 9 February 1990, Cell Press (Cambridge, MA, US) E.R. Fearon et al.: "Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response", pages 397-403, see the whole article (cited in the application)	32-34
Y	---	1,2,4,6 7,9,29
X	Proceedings of the National Academy of Sciences, volume 86, December 1989 (Washington, US) Y. Watanabe et al.: "Exogenous expression of mouse interferon gamma cDNA in mouse neuroblastoma C1300 cells results in reduced tumorigenicity by augmented anti-tumor immunity", pages 9456-9460, see the whole article  --- -/-	35-37
<p><sup>o</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
24-01-1992	12. 02. 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	Maria Peis <i>Maria Peis</i>	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Immunology Today, volume 11, no. 6, June 1990, Elsevier Science Publishers Ltd (GB) S.J. Russell: "Lymphokine gene therapy for cancer", pages 196-200, see especially pages 196-197 ---	32-37
X	Cancer Research, volume 46, no. 9, September 1986, (Philadelphia, US) I.H. Maxwell et al.: "Regulated expression of a diphtheria toxin A-chain gene transfected into human cells: possible strategy for inducing cancer cell suicide", pages 4660-4664, see the abstract; page 4661, lines 10-13 ---	21,22, 24-27
Y	---	1-2,4, 14-16,6 -7,9,29
A	Cancer Research, volume 48, no. 11, 1 June 1988 (Philadelphia, US) E.R. Fearon et al.: "Induction in a murine tumor of immunogenic tumor variants by transfection with a foreign gene", pages 2975-2980, see the whole article, especially page 2979, lines 43-47; page 2980, lines 23-30 (cited in the application) ---	38-40
Y	---	1-2,14- 16
A	Chemical Abstracts, volume 112, no. 19, 7 May 1990 (Columbus, Ohio, US) F.L. Moolten et al.: "Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors", see page 36, abstract 171876a, & J. Natl. Cancer Inst. 1990, 82(4), 297-300 ---	5
A	Biological Abstracts, volume 90, no. 5, 1990, AB-637; S. Ostrand-Rosenberg et al.: "Transfection of major histocompatibility complex class I and class II genes causes tumor rejection", see left-hand column, abstract no. 52893, & J. Immunogenet. 16(4/5) 1989, pages 343-350 -----	

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 28-31, 51-54 are directed to a method of treatment on the animal body the search has been carried out on the alleged effects of the composition.

2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.